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International application number: PCT/EP05/003061

International filing date: 22 March 2005 (22.03.2005)

Document type: Certified copy of priority document

Document details: Country/Office: EP  
Number: 04075892.2  
Filing date: 23 March 2004 (23.03.2004)

Date of receipt at the International Bureau: 23 May 2005 (23.05.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)



World Intellectual Property Organization (WIPO) - Geneva, Switzerland  
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse



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**Patentanmeldung Nr.    Patent application No.    Demande de brevet n°**

04075892.2

Der Präsident des Europäischen Patentamts;  
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets  
p.o.

**R C van Dijk**





Anmeldung Nr:  
Application no.: 04075892.2  
Demande no:

Anmeldetag:  
Date of filing: 23.03.04  
Date de dépôt:

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Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:  
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.  
If no title is shown please refer to the description.  
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Polymeric prodrugs

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s)  
revendiquée(s)  
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/  
Classification internationale des brevets:

A61K47/48

Am Anmeldetag benannte Vertragsstaaten/Contracting states designated at date of  
filing/Etats contractants désignées lors du dépôt:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LU MC NL  
PL PT RO SE SI SK TR LI



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## Polymeric Prodrugs

### Field

- 5 The present invention is directed to polymeric prodrugs having reversible linkages to amino groups of biologically active entities such as peptides, proteins, natural products or synthetic chemical compounds.

### Background

10

Polymers used in drug delivery systems are of interest because the therapeutic efficacy of both many low molecular weight and macromolecular medicinal agents may be significantly improved by combining them with polymers.

- 15 For example, covalent modification of biological molecules with poly(ethylene glycol) (PEG) has been extensively studied since the late 1970s. So-called PEGylated proteins have shown improved therapeutic efficacy by increasing solubility, reducing immunogenicity, and increasing circulation half-life in vivo due to reduced renal clearance and proteolysis by enzymes (Caliceti P., Veronese F.M., Adv. Drug Deliv. Rev. 2003, 55, 1261-1277).

- Many small molecule medicinal agents, like alkaloids and anti-tumor agents, show low solubility in aqueous fluids. One way to solubilize these small molecule compounds is to conjugate them to hydrophilic polymers. A variety of water-soluble  
25 polymers, such as human serum albumin, dextran, lectins, poly(ethylene glycol), poly(styrene-co-maleic anhydride), poly(N-hydroxypropylmethacrylamide), poly(divinyl ether-co-maleic anhydride), hyaluronic acid have been described for this purpose.

- 30 Depot formulations generating long-acting release profiles have been described using non-covalent drug encapsulation. Typically, a drug is mixed with polymer material and processed in such fashion, that the drug is captured in the polymer interior.

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Resulting polymer-protein aggregates may be shaped as microparticles or gels. Drug release occurs if the polymer swells or degradation of the polymer allows for diffusion of the drug to the exterior. Degradation processes may be autohydrolytic or enzyme-catalyzed. Marketed drugs based on non-covalent polymer encapsulation are Lupron Depot and Nutropin Depot. A disadvantage of this approach is that in order to prevent uncontrolled, burst-type release of the non-covalently bound drug, encapsulation has to be highly efficient, requiring strong van der Waals contacts, frequently mediated through hydrophobic moieties, and many conformationally sensitive therapeutics such as proteins or peptides are rendered dysfunctional during the encapsulation process and/or during subsequent storage. Furthermore, dependence of the release mechanism upon biodegradation may cause interpatient variability.

A major challenge in cancer therapy is to selectively target cytotoxic agents to tumor cells. A promising method to accumulate small molecule anticancer agents in tumor tissue and decrease undesirable side effects of these agents is the attachment of the cytotoxin to a macromolecular carrier. The passive targeting of polymeric drug conjugates to tumors is based on the so-called enhanced permeability and retention effect (EPR) as described by Matsumura, Y. and Maeda, H., in Cancer Res., 1986, vol 6, pp 6387-6392. As a result, several polymer-drug conjugates have entered clinical trial as anticancer agents.

However, many medicinal agents are inactive or show decreased biological activity when a polymer is covalently conjugated to the drug molecule. In these cases, a prodrug approach may be applied in such a fashion that the medicinal agent is released from the polymeric carrier in vivo to regain its biological activity. Prodrugs are defined as therapeutic agents that are inactive per se but are predictably transformed into active metabolites (see B. Testa, J.M. Mayer in Hydrolysis in Drug and Prodrug Metabolism, Wiley-VCH, 2003, page 4). The reduced biological activity of the prodrug as compared to the released drug is of advantage if a slow or controlled release of the drug is desired. In this case, a relatively large amount of prodrug may be administered without concomitant side effects and the risk of overdosing. Release of the drug occurs over time, thereby reducing the necessity of repeated and frequent administration of the drug.

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### Definitions established by IUPAC

(as given under <http://www.chem.qmul.ac.uk/iupac/medchem/> (accessed on 8 March 2004))

#### 5 Prodrug

A prodrug is any compound that undergoes biotransformation before exhibiting its pharmacological effects. Prodrugs can thus be viewed as drugs containing specialized non-toxic protective groups used in a transient manner to alter or to eliminate undesirable properties in the parent molecule.

10

#### Double prodrug (or pro-prodrug)

A double prodrug is a biologically inactive molecule which is transformed *in vivo* in two steps (enzymatically and/or chemically) to the active species.

15

#### Carrier-linked prodrug (Carrier prodrug)

A carrier-linked prodrug is a prodrug that contains a temporary linkage of a given active substance with a transient carrier group that produces improved physicochemical or pharmacokinetic properties and that can be easily removed *in vivo*, usually by a hydrolytic cleavage. This is shown graphically in Fig. 1.

20

#### Cascade prodrug

A cascade prodrug is a prodrug for which the cleavage of the carrier group becomes effective only after unmasking an activating group. This is shown graphically in Fig.

25 2.

#### Bioprecursor prodrug

A bioprecursor prodrug is a prodrug that does not imply the linkage to a carrier group, but results from a molecular modification of the active principle itself. This modification generates a new compound, able to be transformed metabolically or chemically, the resulting compound being the active principle.

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### **Biotransformation**

Biotransformation is the chemical conversion of substances by living organisms or enzyme preparations.

- 5 This invention applies in particular to carrier prodrugs and cascade prodrugs. In polymeric prodrugs, the drugs are often linked to a polymeric carrier moiety by a labile bridge formed between the carrier moiety and a hydroxy, amino or carboxy group of the drug molecule.
- 10 Cleavage of a carrier prodrug generates a molecular entity (drug) of increased bioactivity and at least one side product, the carrier. This side product may be biologically inert (for instance PEG) or may have targeting properties (for instance antibodies). After cleavage, the bioactive entity will reveal at least one previously conjugated and thereby masked or protected functional group, and the presence of this
- 15 group typically contributes to the bioactivity. Numerous macromolecular prodrugs are described in the literature where the macromolecular carrier is linked via a labile ester group to the medicinal agent (e.g. Y. Luo, MR Ziebell, GD Prestwich, "A Hyaluronic Acid - Taxol Antitumor Bioconjugate Targeted to Cancer Cells", *Biomacromolecules* 2000, 1, 208-218, J Cheng et al, *Synthesis of Linear, beta-Cyclodextrin Based*
- 20 *Polymers and Their Camptothecin Conjugates*, *Bioconjugate Chem.* 2003, 14, 1007-1017, R. Bhatt et al, *Synthesis and in Vivo Antitumor Activity of Poly(L-glutamic acid) Conjugates of 20(S)-Camptothecin*, *J. Med. Chem.* 2003, 46, 190-193 ; R.B. Greenwald, A. Pendri, C.D. Conover, H. Zhao, Y.H. Choe, A. Martinez, K. Shum, S. Guan, *J. Med. Chem.*, 1999, 42, 3657-3667; B. Testa, J.M; Mayer in *Hydrolysis in*
- 25 *Drug and Prodrug Metabolism*, Wiley-VCH, 2003, Chapter 8) In these cases, the conjugated functional group of the bioactive entity is a hydroxyl group or a carboxylic acid.

- Especially for biomacromolecules but also for small molecule polymer prodrugs, it
- 30 may be desirable to link the macromolecular carrier to amino groups (i.e. N-terminus or lysine amino groups of proteins) of the bioactive entity, as is shown in Fig. 3. This will be the case if masking the drug's bioactivity requires conjugation of a certain amino group of the bioactive entity, for instance an amino group located in an active center or a region or epitope involved in receptor binding. Also, during preparation of

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- the prodrug, amino groups may be more chemoselectively addressed and serve as a better handle for conjugating carrier and drug because of their greater nucleophilicity as compared to hydroxylic or phenolic groups. This is particularly true for proteins which may contain a great variety of different reactive functionalities. In this case
- 5 non-selective conjugation reactions lead to undesired product mixtures which require extensive characterization or purification and may decrease reaction yield and therapeutic efficiency of the product.
- 10 Prodrug activation may occur by enzymatic or non-enzymatic cleavage of the labile bridge between the carrier and the drug molecule, or a sequential combination of both, ie an enzymatic step followed by a nonenzymatic rearrangement.
- In an enzyme-free in vitro environment such as an aqueous buffer solution, almost
- 15 any prodrug will undergo hydrolysis if labile linkages such as ester or amide bonds are present in the molecule. This rate of hydrolysis may be very slow and not therapeutically useful. In an in vivo environment, esterases or amidases are typically present and may cause significant catalytic acceleration of the kinetics of hydrolysis from twofold up to several orders of magnitude (R.B. Greenwald et al. J.Med.Chem.
- 20 1999, 42 (18), 3857-3867).
- The labile bond may be engineered for enzyme selectivity for organ or cell-type targeting. Targeted release of the bioactive entity is effected, if an enzyme, that selectively cleaves the linkage, is specifically present in the organ or cell type chosen
- 25 for treatment. Several examples were published for the prodrug activation of amine containing drugs by specific enzymes. In these cases, targeted release was the objective. G. Cavallaro et al., Bioconjugate Chem, 2001, 12, 143-151 described the enzymatic release of an antitumoral agent by the protease plasmin. Cytarabin was coupled via the tripeptide sequence D-Val-Leu-Lys to the polymer alpha, beta-
- 30 poly(N-hydroxyethyl)-DL-aspartamide (PHEA). Enzymatic release of cytarabin was effected by the protease plasmin which concentration is relatively high in various kinds of tumor mass.

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- Further examples for antitumoral polymeric prodrugs activated by specific enzymes like beta lactamase (R. Satchi-Fainaro et al., Bioconjugate Chem. 2003, 14, 797-804) and cysteine proteases like cathepsin B (R. Duncan et al. J. Contr. Release 2001, 74, 135-146) were described. Wiwattanapatapee et al. (2003) described a dendrimer
- 5 prodrug for colonic delivery of 5-aminosalicylic acid. The drug molecule was conjugated by an azo bond to generation 3 PAMAM dendrimer. 5-aminosalicylic acid is released in the colon by a bacterial enzyme called azo reductase (W. R. Wiwattanapatapee, L. Lomlim, K. Saramunee, J. Controlled Release, 2003, 88: 1-9).
- 10 A.J. Garman et al. (A.J. Garman, S.B. Kalindjian, FEBS Lett. 1987, 223 (2), 361-365 1987) used PEG5000-maleic anhydride for the reversible modification of amino groups in tissue-type plasminogen activator and urokinase. Regeneration of functional enzyme from PEG-uPA conjugate upon incubation at pH 7.4 buffer by cleavage of the maleamic acid linkage followed first order kinetics with a half-life of 6.1 h. The
- 15 prodrug cleavage was not investigated in the presence of enzymes, and it can be expected – as explained above – that in vivo proteases will significantly contribute to the cleavage of the labile amide linkage. A further disadvantage of this linkage is the lack of stability of the conjugate at lower pH values that limits this approach to active agents stable at basic pH values as purification of the active agent polymer conjugate
- 20 has to be performed under basic conditions.
- R.B. Greenwald, A. Pendri, C.D. Conover, H. Zhao, Y.H. Choe, A. Martinez, K. Shum, S. Guan, J. Med. Chem., 1999, 42, 3657-3667 & PCT Patent Application WO-A-99/30727 described a methodology for synthesizing poly(ethylene glycol) prodrugs
- 25 of amino-containing small molecule compounds based on 1,4- or 1,6-benzyl elimination. In this approach the amino group of the drug molecule is linked via a carbamate group to a PEGylated benzyl moiety. The poly(ethylene glycol) was attached to the benzyl group by ester, carbonate, carbamate, or amide bonds. The release of PEG from the drug molecule relied on the enzymatic cleavage of these
- 30 masking groups. The cleavage of the release-triggering masking group is followed in this approach by the classical and rapid 1,4- or 1,6-benzyl elimination. As a consequence the highly reactive and potentially toxic quinone methide or quinonimine methide is liberated.

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This is an example for a cascade carrier prodrug where the masking group is identical with the carrier and is shown diagrammatically in Fig. 4.

5 This linker system was also used for releasable poly(ethylene glycol) conjugates of proteins (S. Lee, R.B. Greenwald et al. Bioconj. Chem. 2001, 12 (2), 163-169).  
Lysozyme was used as model protein because it loses its activity when PEGylation takes place on the epsilon-amino group of lysine residues. Various amounts of PEG Linker were conjugated to the protein. Regeneration of native protein from the PEG conjugates occurred by enzymatic cleavage in rat plasma or in non-physiological high  
10 pH buffer.

Greenwald et al. published in 2000 a poly(ethylene glycol) drug delivery system of amino-containing prodrugs based on trimethyl lock lactonization (R.B. Greenwald et al. J. Med. Chem. 2000, 43(3), 457-487; PCT Patent Application No. WO-A-  
15 02/089789). In this prodrug system substituted o-hydroxyphenyl-dimethylpropionic acid is coupled to amino groups of drug molecules by an amide bond. The hydroxy group is linked to PEG by an ester, carbonate, or carbamate group. The rate determining step in drug release is the enzymatic cleavage of these functional groups followed by fast amide cleavage by lactonization, liberating a potentially toxic  
20 aromatic lactone side product.

A common disadvantage of the linker systems described by Greenwald is the release of a potentially toxic aromatic small molecule side product after cleavage of these prodrug linker molecules.

25

More recently, R.B. Greenwald et al. (Greenwald et al. J. Med. Chem. 2004, 47, 726-734) described a PEG prodrug system based on bis-(N-2-hydroxyethyl)glycin amide (bicin amide) linker. In this system two PEG molecules are linked to a bicin molecule coupled to an amino group of the drug molecule. The first two steps in prodrug  
30 activation is the enzymatic cleavage of both PEG molecules. Different linkages between PEG and bicin are described resulting in different prodrug activation kinetics. The main disadvantage of this system is the slow hydrolysis rate of bicin amide conjugated to the drug molecule ( $t_{1/2} = 3$  h in phosphate buffer) resulting in

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the release of a bicin-modified prodrug intermediate that may show different pharmacokinetic and pharmacodynamic properties than the parent drug molecule.

5 The in vivo release of stable linker-drug intermediates or potentially toxic side products can be avoided, if the carrier is not serving as a masking group and the activating group is coupled to the carrier by means of a stable bond. Antczak et al. (Bioorg Med Chem 9 (2001) 2843-48) describe a reagent which forms the basis for a cascade prodrug system for amine-containing drug molecules. In this approach an antibody serves as carrier, a stable bond connects the antibody to an activating moiety,  
10 carrying an enzymatically cleavable masking group. Upon enzymatic removal of the masking group, a second labile bond cleaves and releases the drug compound, as is shown in Fig. 5.

15 A drawback of predominantly enzymatic cleavage is interpatient variability. Enzyme levels may differ significantly between individuals resulting in biological variation of prodrug activation by enzymatic cleavage. Enzyme levels may also vary depending on the site of administration, for instance it is known that in the case of subcutaneous injection, certain areas of the body yield more predictable therapeutic effects than others. To reduce this unpredictable effect, non-enzymatic cleavage or intramolecular  
20 catalysis is of particular interest (see, for example, B. Testa, J.M. Mayer in Hydrolysis in Drug and Prodrug Metabolism, Wiley-VCH, 2003, page 5).

Using enzyme specificity to control both targeting and release kinetics is a compromise. Enzyme-dependent release results in interpatient variability. Targeting  
25 through enzyme selectivity is of lower efficiency than for instance targeting through antibodies and may be achieved by more reliable means for instance by introducing certain structural features into the polymer moiety of the prodrug.

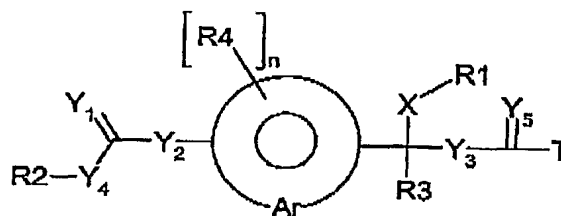
30 For these reasons, there is a need to provide novel linker and/or carrier technologies for forming polymeric prodrugs of amine containing active agents in order to overcome the limitations of the described polymeric prodrugs.

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### Detailed description of the invention

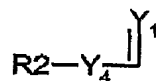
The present invention addresses the disadvantages described above. The invention provides for a polymeric prodrug of Formula I.



Formula I

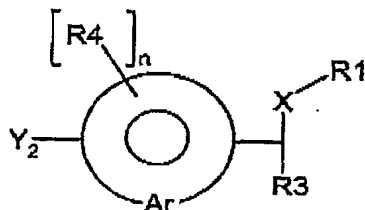
wherein  $Y_1$  to  $Y_5$ ,  $R_1$  to  $R_4$ ,  $T$ ,  $X$  and  $Ar$  are defined below:

Native drug release is effected by a two step mechanism. The first step is the rate-determining cleavage of the masking group:



from the polymeric prodrug in vivo.

As described above, cleavage of the masking moiety may be mediated by an enzymatic or a non-enzymatic step, such as pH-dependent hydrolysis or intramolecular cyclization. In the preferred embodiment of the invention, cleavage is effected non-enzymatically. The second and final step in release of the regenerated native drug is the fast, spontaneous and irreversible so-called 1,4- or 1,6 or 1,(4+2n) with  $n=2, 3, 4$  or higher elimination of the

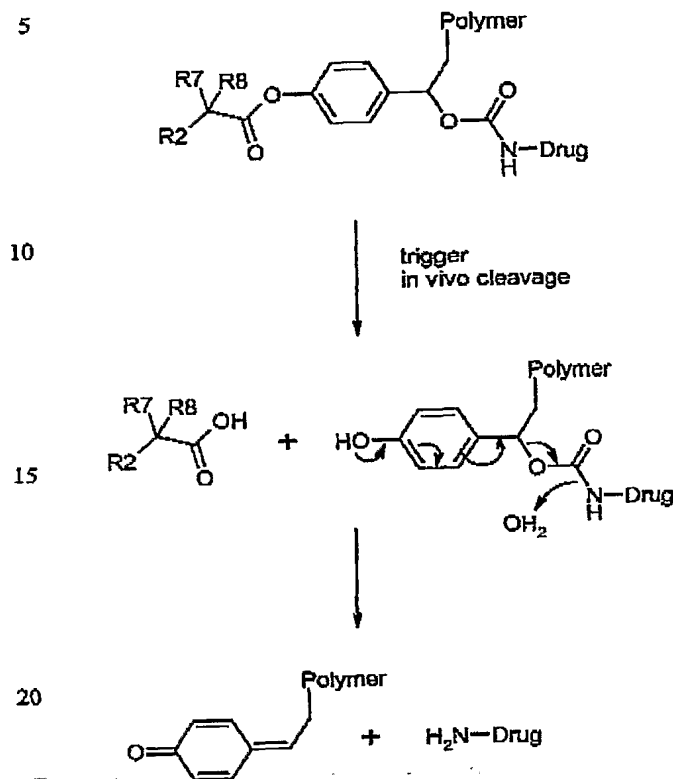


moiety of the remaining polymeric prodrug.

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This mechanism of native drug release from a polymeric prodrug triggered by hydrolytic cleavage of the masking group followed by a 1,6-elimination step of the activating group is exemplified by an example of a polymeric prodrug according to the present invention.



25 Definition of  $Y_1$  to  $Y_5$ ,  $R_1$  to  $R_4$ ,  $T$ ,  $X$  and  $Ar$  in formula I

$T$  is  $D$  or  $A$

and  $D$  is a residue of an amine-containing biologically active material including but not limited to small molecule bioactive agents or biopolymers like proteins, polypeptides and oligonucleotides (RNA, DNA), peptide nucleic acids (PNA),

30

and  $A$  is a leaving group.

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Non-limiting examples of suitable leaving groups A include chloride, bromide, fluoride, nitrophenoxy, imidazolyl, N-hydroxysuccinimidyl, N-hydroxybenzotriazolyl, N-hydroxyazobenzotriazolyl, pentafluorophenoxy, N-hydroxysulfosuccinimidyl, or any other leaving group known by those skilled in the art.

Suitable organic small molecule bioactive drugs include, without limitation, moieties such as central nervous system-active agents, anti-infective, anti-neoplastic, antibacterial, anti-fungal, analgesic, contraceptive, anti-inflammatory, steroidal, vasodilating, vasoconstricting, and cardiovascular agents with at least one primary or secondary amino group. Non-exclusive examples of such compounds are daunorubicin, doxorubicin, idarubicin, mitoxantron, aminoglutethimide, amantadine, diaphenylsulfon, ethambutol, sulfadiazin, sulfamerazin, sulfamethoxazol, sulfalen, cinafloxacin, moxifloxacin, ciprofloxacin, enoxacin, norfloxacin, neomycin B, spectinomycin, kanamycin A, meropenem, dopamin, dobutamin, lisinopril, serotonin, carbutamid, acivicin, etc.

Suitable proteins and polypeptides having at least one free amino group include but are not limited to ACTH, adenosine deaminase, agalsidase, albumin, alfa-1 antitrypsin (AAT), alfa-1 proteinase inhibitor (API), alteplase, anistreplase, anicod serine protease, antibodies (monoclonal or polyclonal, and fragments or fusions), antithrombin III, antitrypsins, aprotinin, asparaginases, biphalin, bone-morphogenic proteins, calcitonin (salmon), collagenase, DNase, endorphins, enfuvirtide, enkephalins, erythropoietins, factor VIIa, factor VIII, factor VIIIa, factor IX, fibrinolysin, fusion proteins, follicle-stimulating hormones, granulocyte colony stimulating factor (G-CSF), galactosidase, glucagon, glucocerebrosidase, granulocyte macrophage colony stimulating factor (GM-CSF), phospholipase-activating protein (PLAP), gonadotropin chorionic (hCG), hemoglobins, hepatitis B vaccines, hirudin, hyaluronidases, iduronidase, immune globulins, influenza vaccines, interleukins (1 alfa, 1 beta, 2, 3, 4, 6, 10, 11, 12), IL-1 receptor antagonist (rhIL-1ra), insulins, interferons (alfa 2a, alfa 2b, alfa 2c, beta 1a, beta 1b, gamma 1a, gamma 1b), keratinocyte growth factor (KGF), transforming growth factors, lactase, leuprolide, levothyroxine, luteinizing hormone, Lyme vaccine, natriuretic peptide, pancrelipase, papain, parathyroid hormone, PDGF, pepsin, platelet activating factor acetylhydrolase



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(PAF-AH), prolactin, protein C, octreotide, secretin, somatostatin, superoxide dismutase (SOD), somatropins (growth hormone), streptokinase, sucrase, tetanus toxin fragment, tilactase, thrombins, thymosin, thyroid stimulating hormone, thyrotropin, tumor necrosis factor (TNF), TNF receptor-IgG Fc, tissue plasminogen activator (tPA), TSH, urate oxidase, urokinase, vaccines, plant proteins such as lectins and ricins.

Also included herein is any synthetic polypeptide or any portion of a polypeptide with in vivo bioactivity. Furthermore, proteins prepared by recombinant DNA methodologies including mutant versions of aforementioned proteins, antibody fragments, single chain binding proteins, catalytic antibodies and fusion proteins are included.

Preferred proteins are antibodies, calcitonin, G-CSF, GM-CSF, erythropoietins, hemoglobins, interleukins, insulins, interferons, SOD, somatropin, TNF, TNF-receptor-IgG Fc.

X is a spacer moiety such as R5-Y6

Y<sub>1</sub>, Y<sub>2</sub> can each be either O, S, or NR6, independently of each other.

Y<sub>3</sub>, Y<sub>5</sub> can each be either O or S, independently of each other.

Y<sub>4</sub> is O, NR6, or -C(R7)(R8)-

Y<sub>6</sub> is O, S, NR6, succinimide, maleimide, unsaturated carbon-carbon bonds or any heteroatom containing a free electron pair.

R<sub>3</sub> is selected from hydrogen, substituted or non-substituted linear, branched or cyclical alkyl or heteroalkyl, aryls, substituted aryls, substituted or non-substituted heteroaryl, cyano, nitro, halogen, carboxy, carboxyalkyl, alkylcarbonyl, carboxamidoalkyl, etc.

The term "heteroalkyl" in the context of the present invention denotes (linear, cyclical or branched) alkyl chains where the alkyl chains contain one or more heteroatoms, selected independently from O, S, N, P, Si, etc. or groups, selected independently from amide, ester, phosphonate ester, phosphate ester, double or triple bonds, carbamate, urea, thiourea, thiocarbamate, oxime, etc.

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Each R4 substitution on Ar may be the same or different and is selected independently from hydrogen, substituted or non-substituted linear, branched or cyclical alkyl or heteroalkyl, aryl, substituted aryl, substituted or non-substituted heteroaryl,  
5 substituted or non-substituted linear, branched, or cyclical alkoxy, substituted or non-substituted linear, branched, or cyclical heteroalkyloxy, aryloxy, heteroaryloxy, etc.

R4 is selected preferably from small substituents such as hydrogen, methyl, ethyl, ethoxy, methoxy, and other C1 to C6 linear, cyclical or branched alkyls and  
10 heteroalkyls.

n is zero or a positive integer.

R7 and R8 are selected from hydrogen, substituted or non-substituted linear, branched  
15 or cyclical alkyl or heteroalkyl, aryls, substituted aryls, substituted or non-substituted heteroaryl, carboxyalkyl, alkylcarbonyl, carboxamidoalkyl, cyano, halogen, etc.

R5 is selected from substituted or non-substituted linear, branched or cyclical alkyl or heteroalkyl, aryls, substituted aryls, substituted or non-substituted heteroaryl,  
20 carboxyalkyl, alkylcarbonyl, carboxamidoalkyl, etc.

R6 is selected from hydrogen, substituted or non-substituted linear, branched or cyclical alkyl or heteroalkyl, aryls, substituted aryls, substituted or non-substituted heteroaryl, etc.  
25

R1 is a polymer.

Non-limiting examples for suitable polymers are polyalkyloxy-based polymers like poly(propylene glycol) or poly(ethylene glycol), dextran, chitosan, hyaluronic acid and derivatives, alginate, agarose, cellulose, hydroxyethyl starch (HES) and other  
30 carbohydrate-based polymers, poly(vinyl alcohols), poly(oxazolines), poly(anhydrides), poly(ortho esters), poly(carbonates), poly(urethanes), poly(acrylic acids), poly(acrylamides) such as hydroxypropylmethacrylamide (HMPA), poly(organophosphazenes), poly(vinylpyrrolidone), poly(cyanoacrylates), poly(esters) such as poly(lactic acid) or poly(glycolic acids), poly(iminocarbonates), poly(amino

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acids) such as poly(glutamic acid), collagen, gelatin, copolymers, grafted copolymers, cross-linked polymers, and block copolymers from the above listed polymers.

- 5 Further examples include branched and hyperbranched polymers. Examples for such polymers include dendrimers and other dense star polymers. (R. Esfand, D.A. Tomalia, Drug Discov Today, 2001, 6(8), 427-436; P.M. Heegaard, U. Boas, Chem. Soc. Rev. 2004 (33(1), 43-63; S.M. Grayson, J.M. Frechet, Chem. Rev. 2001, 101 (12), 3819-3868).

10

R1 can also be a biopolymer like a protein. Non-limiting examples include albumin, antibodies, fibrin, casein, and other plasma proteins.

- Each polymer can carry one or more biologically active substances linked to the polymer by the prodrug linker described herein or any other linker known to the person skilled in the art. The polymers may have further substituents and may be functionalized for attachment to the spacer moiety X. Non-limiting examples of such functional groups comprise carboxylic acid and activated derivatives, amino, maleimide, thiol, sulfonic acid and derivatives, carbonate and derivatives, carbamate and derivatives, hydroxyl, aldehyde, ketone, hydrazine, isocyanate, isothiocyanate, phosphoric acid and derivatives, phosphonic acid and derivatives, haloacetyl, alkyl halides, acryloyl, arylating agents like aryl fluorides, hydroxylamine, disulfides like pyridyl disulfide, vinyl sulfone, vinyl ketone, diazoalkanes, diazoacetyl compounds, epoxide, oxirane, and aziridine.

25

Preferred functional groups for the polymer include but are not limited to thiol, maleimide, amino, carboxylic acid and derivatives, carbonate and derivatives, carbamate and derivatives, aldehyde, and haloacetyl.

- 30 Especially preferred functional groups include thiol, maleimide, amino, carboxylic acid and derivatives, carbamate and derivatives, and carbonate and derivatives thereof.

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Non-limiting examples for suitable bonds or groups formed between X and R1 include disulfide, S-succinimido, amide, amino, carboxylic ester, sulfonamide, carbamate, carbonate, ether, oxime, hydrazone, urea, thiourea, phosphate, phosphonate, etc.

5

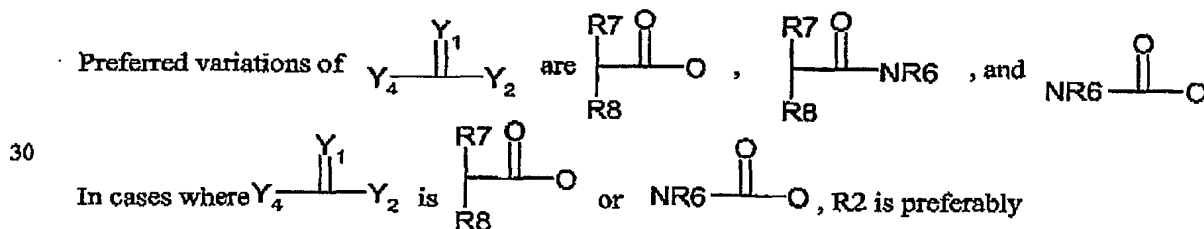
Preferred bonds or groups formed between X and R1 comprise S-succinimido, amide, carbamate, and urea.

Preferably, the polymers are well hydrated, degradable or excretable, and nontoxic and non-immunogenic in mammals. Preferred polymers include polyalkoxy-based polymers like polyethylene glycol and polyethylene glycol reagents as those described in Nektar Inc. 2003 catalog "Nektar Molecule Engineering – Polyethylene Glycol and Derivatives for Advanced PEGylation" and branched, hyperbranched or cross-linked polymers.

15

R2 is selected from hydrogen, substituted or non-substituted linear, branched or cyclical alkyl, aryls, substituted aryls, substituted or non-substituted linear, branched or cyclical heteroalkyl, substituted or nonsubstituted heteroaryls, substituted or non-substituted linear, branched, or cyclical alkoxy, substituted or non-substituted linear, branched, or cyclical heteroalkyloxy, aryloxy, heteroaryloxy, carboxy, carboxyalkyl, alkylcarbonyl, carboxamidoalkyl, peptide sequences which can be cleaved by specific enzymes, etc.

R2 is selected preferably from non-toxic substituted or non-substituted linear, branched or cyclical alkyls or heteroalkyls.



selected from substituted linear, branched or cyclical alkyl or heteroalkyl, containing preferably a nucleophile that can perform a nucleophilic attack at the carbonyl carbon and thus catalyse the cleavage of the masking group by intramolecular catalysis or

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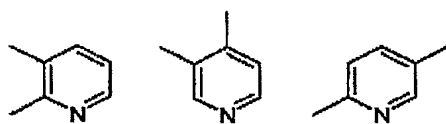
cyclization. Nucleophiles are any heteroatoms carrying at least one free electron pair. Preferred nucleophiles include amino, thiol, carboxylic acid, hydroxylamine, hydrazine. The nucleophile is situated preferably four to fifteen atoms away from  $Y_2$ .  $R_7$  and/or  $R_8$  are preferably not hydrogen.

5

Figure 8 shows an example where cleavage of the masking group in formula I is by intramolecular cyclization.

Ar of formula I is a multi-substituted aromatic hydrocarbon or a multi-substituted aromatic heterocycle. To be aromatic, the number of pi electrons must satisfy the Hückel rule ( $4n+2$ ) and the cycle has to be planar. A huge variety of compounds satisfy these criteria and thus are suitable as Ar in formula I. Non-limiting preferred aromatic moieties include:

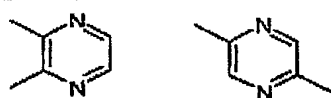
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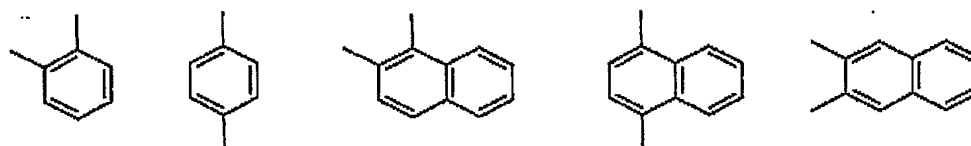
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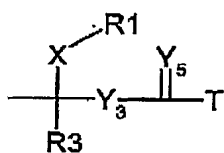
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wherein W is O, N, or S, independent from each other.

- 5  $\text{Y}_2$  and  have to be arranged on the aromatic ring in such a fashion that a 1,4- or 1,6- or 1,(4+2n), with n= 2, 3, 4 and higher, elimination can take place (see above). For example, in the case of a 6-membered ring, the substituents have to be arranged *ortho* or *para*.

- 10 Preferred moieties for Ar are mono- and dicyclic aromatic hydrocarbons or aromatic heterocycles.

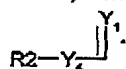
Especially preferred moieties are monocyclic five- or six-membered aromatic hydrocarbons or aromatic heterocycles.

15

### General synthesis procedures of the polymeric prodrugs

- 20 Synthesis of representative examples of polymeric prodrugs according to the present invention is described in the Examples section.

However, prodrugs of the present invention can be prepared in various different fashions. Figure 9 shows general routes for the synthesis of the polymeric prodrugs of the present invention.

- 25 In a first method, intermediate (III) is provided by acylating  $\text{Y}_2$  of starting material (II) with .

- 30 For this, X may have to be protected with a reversible protecting group  $\text{PG}_1$ . Suitable protecting groups are described in TW Greene, P.G.M. Wuts, Protective groups in organic synthesis, 1999, John Wiley & Sons, 3<sup>rd</sup> ed.. From intermediate (III) two alternative routes can be used to yield (Ia). In a first route intermediate (III) is activated by an activating agent such as 4-nitrophenyl chloroformate or disuccinyl carbonate to yield (IV). The amine containing drug molecule is attached to (IV) to yield (V) by displacing the leaving group of the activated intermediate (IV). After

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deprotection of X such as by treating intermediate (V) with reagents like trifluoroacetic acid or DTT (where applicable) deprotected intermediate (V) is then reacted with polymer R1 to yield the polymeric prodrug (Ia).

5 In a second route the polymer R1 is first attached to the intermediate (III) after deprotection of X (where applicable) to form intermediate (VI). After an activation step intermediate (VII) is formed. (VII) is reacted with the amine containing drug molecule to form the polymeric prodrug (Ia).

10 In a second method, intermediate (VIII) is provided by activating starting material (II) by an activating agent such as 4-nitrophenyl chloroformate. For this, Y<sub>2</sub> and/or X may have to be protected with a protecting group PG<sub>2</sub> and/or PG<sub>1</sub>. Amine containing drug is reacted with intermediate (VIII) to form (IX). In a first route, Y<sub>2</sub> of (IX) is selectively deprotected and acylated to form intermediate (V) which is further processed to (Ia) as described above. In a second route X is selectively deprotected  
15 and reacted with polymer R1 to form intermediate (X). Y<sub>2</sub> of (X) is then deprotected and acylated to form the polymeric prodrug (Ia).

In a third method starting material (II) is reacted with polymer R1 to form intermediate (XI). In one route, intermediate (XI) can be acylated to form  
20 intermediate (VI) which processed as described above to form polymeric prodrug (Ia). In a second route, Y<sub>2</sub> is protected by the protecting group PG<sub>2</sub>, activated and reacted with the amine containing drug molecule to form (X). Intermediate (X) is then processed as described above to form the polymeric prodrug (Ia).

25 For all methods described, further functional groups such as Y<sub>3</sub> or nucleophiles present in R2 may have to be protected with suitable protecting groups.

It is understood, that linker structures according to the outlined invention and carrying protecting groups or leaving groups as described and used in the synthesis of  
30 corresponding polymeric prodrugs are considered within the range of the invention.

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### Application of the polymeric prodrugs in molecular therapy

One advantage of the described prodrug systems is to enable depot formulations without the need for encapsulation. Until now, many biocompatible materials could  
5 not be used for depot formulations due to their lack of encapsulation properties. From such well-hydrated and mechanically soft biocompatible materials, drugs would be released too fast for most therapeutic applications. In combination with the prodrug linkers described in this invention, the carrier material may be optimized for its biocompatibility properties as the release is solely governed by the linker cleavage  
10 kinetics and does not require chemical or enzymatic degradation of the polymer carrier itself.

A second advantage of the polymeric prodrugs of the present invention is their predominantly non-enzymatic cleavage: the half-life of the prodrug in suitably  
15 buffered human blood plasma of pH 7.4 (with aqueous buffer concentration < 50 %) is at least 50 % of the half-life of the prodrug in enzyme-free buffer pH 7.4.

This feature allows for better predictability and control of release rates after administration to a living organism and reduces interpatient variability.  
20

Release rates are governed by a substantially chemical reaction which is in turn dependent on the molecular structure of the linker. Systematic or random modifications of the chemical structure, for instance by changing substituents in one or more positions, for instance a masking group in a cascade prodrug, allows for the  
25 generation of linkers with differing release rates. It is therefore possible to create a variety of linkers and select those fast or slow cleaving linkers according to the demands posed by a given medicinal or therapeutic application.

One embodiment of the invention relates to such cascade-prodrugs where removal of  
30 a masking group from an activating moiety triggers a release sequence.

Another advantageous feature which is part of this invention is the attachment of the polymer carrier through a stable covalent bond to an activating moiety involved in a double or cascade prodrug release mechanism. Typically in polymer pro-prodrugs, a



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bond between the carrier group and the activating moiety is cleaved, this being the release-controlling step, followed by a more rapid molecular rearrangement of the activating moiety and cleavage of a second labile linkage which is located between the activating moiety and the drug. After completion of this sequence, the carrier as well as the activating moiety are released in addition to the drug. Due to the rearrangement, the activating moiety is released in a highly reactive form and may cause direct damage to surrounding biomolecules, or potentially toxic derivatives of this moiety may be formed in vivo. As part of this invention, the activating moiety remains attached to the carrier after drug release and therefore cannot diffuse into the environment. Carrier attachment greatly reduces the reactivity of the moiety and the probability of unwanted side effects.

In contrast to the enzymatic dependency of masking group removal as described by Antczak, C., Bauvois, B., Monneret, C., Florent J-C, Bioorg Med Chem 9, 2001, 2843), a higher level of control over release rates can be achieved if the masking group has enzyme-independent, self-eliminating properties. For instance the masking group may contain an amine group attached to a four carbon atom chain, which in turn is linked through an ester group to the activating moiety. The free amine may react with this ester in an intramolecular fashion, resulting in ester cleavage, amidation and cyclization of the masking group, forming a six-membered ring. Structural features of this masking group such as nucleophilicity of the amine group and ring-forming capacity may be systematically optimized in order to precisely adjust the rate of prodrug cleavage. Such intramolecular reactions resulting in unmasking and subsequent rearrangement are highly independent from enzymes due to the fact that intramolecular reactions are generally preferred over intermolecular reactions and are shown diagrammatically in Fig.6.

In another embodiment of the invention, independency of prodrug cleavage from enzyme levels is achieved by providing a prodrug containing a sterically demanding carrier group as is shown in Fig. 7.

Such encapsulation or sterical protection by the sterically demanding carrier group may be conferred by a branched, hyperbranched, crosslinked or self-assembled structure of the carrier polymer. Such polymers tend to form a densely packed

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molecular volume, as exemplified for instance in dendrimers, dense star polymers or bead-shaped nano- and microparticles or amorphous gels. If the linkage of such a carrier to the drug is located in the interior of such a carrier polymer, the linked drug will be efficiently encapsulated and protected from enzymatic attack. In this case, 5 sterical hindrance by the polymer prevents enzymes from accessing and cleaving the labile linkage.

In yet another embodiment, enzyme-independent prodrug cleavage is achieved by combining an intramolecular self-eliminating masking group with an encapsulating 10 hyperbranched or crosslinked or self-assembled carrier.

#### Description of the Figures

- 15 Fig. 1 shows a carrier-linked prodrug.  
Fig. 2 shows a cascade prodrug.  
Fig. 3 shows cleavage of a prodrug with an amino group.  
Fig. 4 shows prodrug cleavage from a carrier which is identical with a masking group.  
Fig. 5 shows cleavage of a cascade prodrug system  
20 Fig. 6 shows cleavage and intramolecular reaction of the masking group.  
Fig. 7 shows cleavage of a prodrug having a sterically demanding carrier group.  
Fig. 8 shows cleavage of the masker group by intramolecular cyclisation.  
Fig. 9 shows general synthesis methods.

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## Examples

### Materials

Fmoc-amino acids, resins and PyBop were purchased from Novabiochem and are named according to the catalogue. Fmoc-Ado-OH was obtained from Neosystem. All additional chemicals were purchased from Sigma Aldrich. Recombinant human insulin was from ICN Biomedicals (USA). Maleimide-PEG5k was obtained from Nektar (USA). 5-(and-6)-carboxyfluorescein succinimidyl ester (mixed isomers) was obtained from Molecular Probes.

### Solid phase synthesis reaction medium

Solid phase synthesis was performed on TentaGel Sieber amide resin with a loading of 0.17 mmol/g. Syringes equipped with polypropylene frits were used as reaction vessels.

### Standard coupling cycle for fmoc-protected amino acids

For fmoc protecting-group removal, the resin was repeatedly (three times, 4 min each) agitated with 2/2/96 (v/v/v) piperidine/DBU/DMF and repeatedly (six times) washed with DMF.

Coupling of fmoc-protected amino acids to free amino groups on resin was achieved by agitating the resin with 3 equivalents (eq) of Fmoc-amino acid, 3 eq PyBop and 6 eq DIEA in relation to free amino groups in DMF for 60 min. Finally, the resin was repeatedly (five times) washed with DMF.

### Standard cleavage protocol for TentaGel Sieber amide resin

Upon completed synthesis, the resin was washed with DCM, dried in vacuo and treated repeatedly (five times) with 97/2/1 (v/v) DCM/TES/TFA. After evaporation, compounds were purified by preparative RP-HPLC (Waters 600).

### Analysis

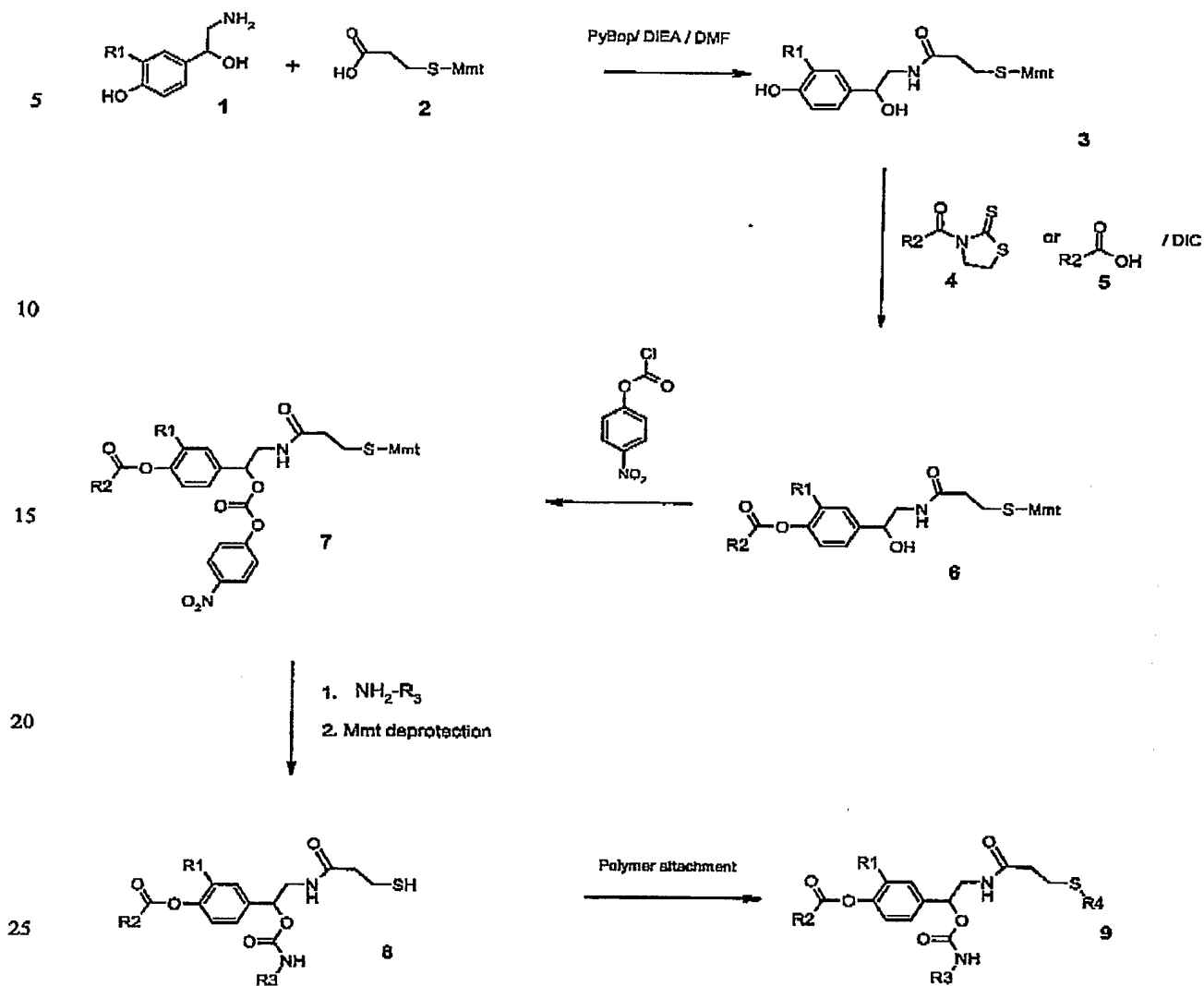
Mass spectrometry (MS) was performed on a Waters ZQ 4000 ESI instrument and spectra were, if necessary, interpreted by Waters software MaxEnt.

Size exclusion chromatography was performed using an Amersham Bioscience AEKTAbasic system equipped with a Superdex 200 column (Amersham Bioscience).

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### Overview - synthesis of polymeric prodrugs



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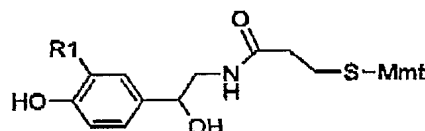
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### Synthesis of compound 2

Mmt-chloride (1 eq) and mercaptopropionic acid (1.1 eq) were dissolved in TFA and incubated for 30 min. The solvent was removed under reduced pressure. The product  
5 was dissolved in pyridine, diluted in water, acidified by acetic acid and extracted in ether. The ether phase was separated and dried over Na<sub>2</sub>SO<sub>4</sub>. Solvent was removed under reduced pressure and product 2 was purified by RP-HPLC.

### Synthesis of compounds 3a and 3b

10

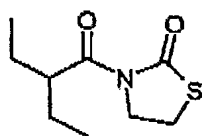
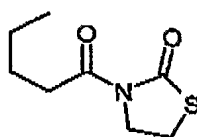
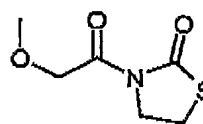
15 **3a** R<sup>1</sup> = H**3b** R<sup>1</sup> = OMe

Octopamine hydrochloride **1a** or normetanephrine hydrochloride **1b** (2 eq), DIEA (4 eq), and PyBOP (1 eq) were dissolved in DMF, 1 eq 2 was added and the mixture was reacted for 50 min at room temperature. After addition of acetic acid (7 eq) the  
20 products **3a** or **3b** were purified by RP-HPLC.

**3a**: MS [M+Na]<sup>+</sup> = 536 (MW+Na calculated = 536.2 g/mol)**3b**: MS [M+Na]<sup>+</sup> = 566 (MW+Na calculated = 566.2 g/mol)

### 25 Synthesis of mercaptothiazolides 4a, 4b, and 4c

30

**4a****4b****4c**

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#### General synthesis protocol:

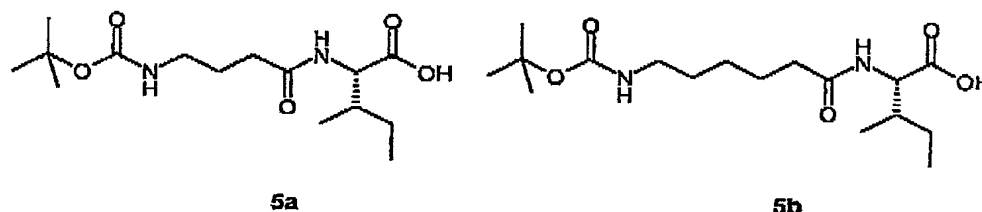
2-Mercaptothiazoline and triethylamine (1.5 eq) were dissolved in dry THF and the respective acid chloride (1 eq) was added. The mixtures were stirred for 1 h at 50 °C under inert gas atmosphere and cooled to room temperature. 0.5 N aqueous HCl was added and the separated organic phases were dried over Na<sub>2</sub>SO<sub>4</sub>. After concentration in vacuo the residues were purified by silica gel column chromatography using heptane/acetic acid ester (1/1) as mobile phase. All mercaptothiazolides were collected as viscous yellow oils.

4a R<sub>f</sub> (heptane/acetic acid ester 1:1) = 0.65

4b R<sub>f</sub> (heptane/acetic acid ester 1:1) = 0.7

4c R<sub>f</sub> (heptane/acetic acid ester 1:1) = 0.35

#### Synthesis of compounds 5a and 5b



#### General synthesis protocol:

1 g 2-chlorotrityl chloride resin (loading 1.6 mmol/g) was incubated for 1 h with 850 mg (2.4 mmol) Fmoc-Isoleucine and 840 µl (4.8 mmol) DIEA in 5 ml 1/1 DCM/DMF. After fmoc removal and washing of the resin with DMF, Boc-aminobutyric acid or boc-aminohexanoic acid, respectively, were coupled to 0.5 g resin each according to the standard coupling cycle for fmoc-protected amino acids. Compounds 5a or 5b, respectively, were cleaved from the resin with 97/1/2 (v/v) DCM/TFA/TES for 45 min. After neutralisation with pyridin, solvents were removed under reduced pressure and 5a or 5b were purified by RP-HPLC.

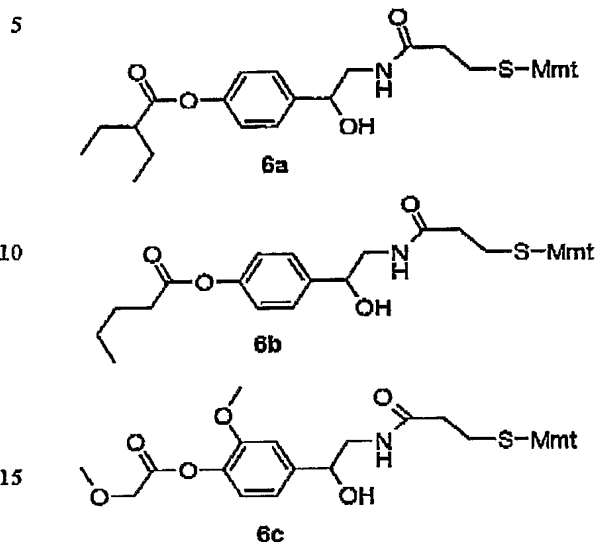
5a MS [M+Na]<sup>+</sup> = 339.2 (MW+Na calculated = 339.4 g/mol)

5b MS [M+Na]<sup>+</sup> = 367.4 (MW+Na calculated = 367.5 g/mol)

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Synthesis of compounds 6a, 6b, and 6c



General synthesis protocol:

20 Mercaptothiazolidine 4a or 4b (1 eq), phenol 3a (4 eq) and DMAP (4 eq) were refluxed in DCM for 2 h under nitrogen atmosphere. After neutralization with acetic acid, the solutions were concentrated and the crude products 6a or 6b, respectively, were purified by RP-HPLC. 6c was prepared as described above using starting materials 4c and 3b.

25

6a MS  $[M+Na]^+ = 634$  (MW+Na calculated = 634.3 g/mol)

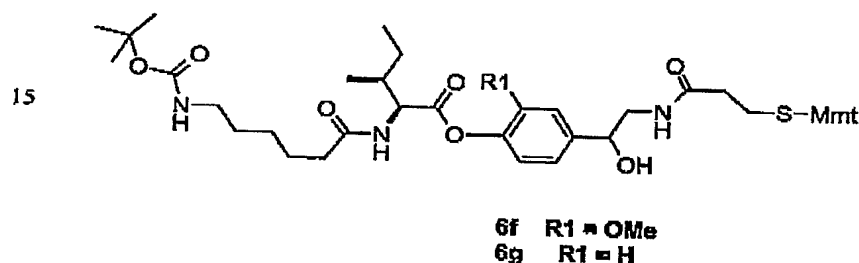
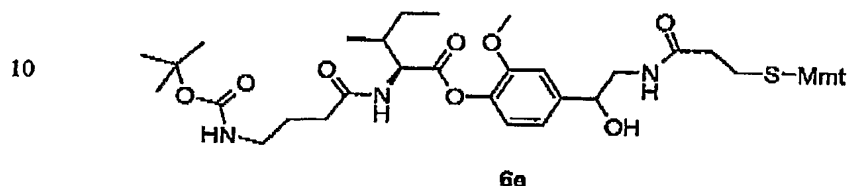
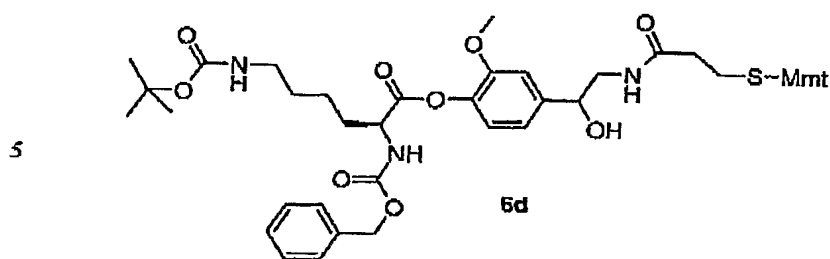
6b MS  $[M+Na]^+ = 620$  (MW+Na calculated = 620.3 g/mol)

6c MS  $[M+Na]^+ = 638$  (MW+Na calculated = 638.4 g/mol)

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# Synthesis of compounds 6d to 6g



## 20 General synthesis protocol:

Carboxylic acid **5a**, **5b** or Z-Lys(Boc)-OH (1 eq), phenol **3b** (1 eq), DIC (1 eq), and DMAP (2 eq) in DMF were reacted for 1h at room temperature. After addition of acetic acid (4 eq) the resulting carboxylic esters **6d**, **6e** or **6f**, respectively, were purified by RP-HPLC.

25 **6g** was synthesized as described above using **5b** and **3a** as starting materials.

**6d** MS  $[M+Na]^+ = 928$  (MW+Na calculated = 928.6 g/mol)

**6e** MS  $[M+Na]^+ = 864$  (MW+Na calculated = 864.5 g/mol)

30 **6f** MS  $[M+Na]^+ = 892$  (MW+Na calculated = 892.6 g/mol)

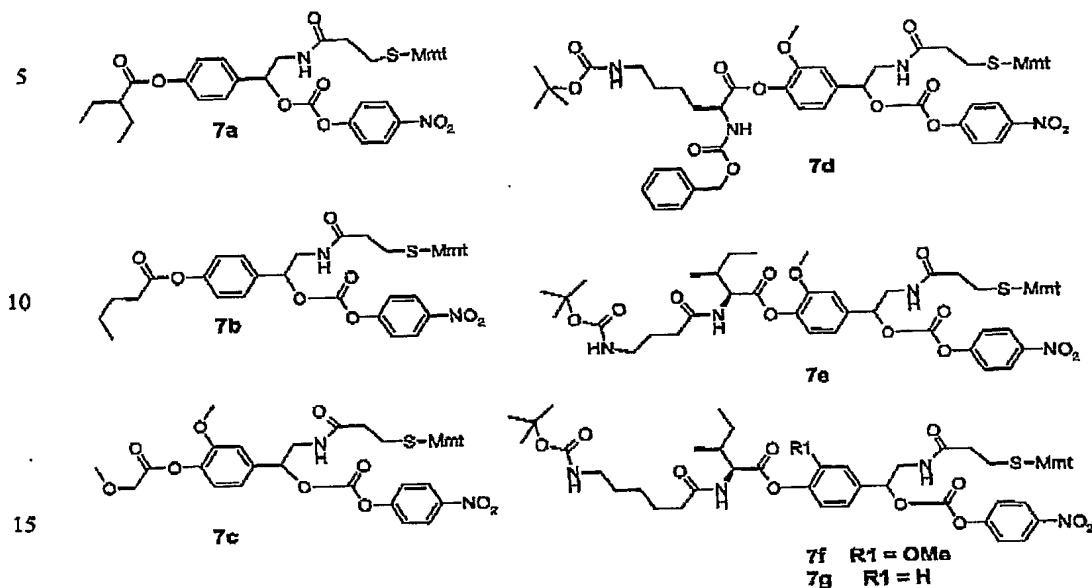
**6g** MS  $[M+Na]^+ = 862$  (MW+Na calculated = 862.6 g/mol)



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# Synthesis of compounds 7a to 7g



## General synthesis protocol:

20 Alcohols 6a, 6b, 6c, 6d, 6e, 6f or 6g (1 eq), 4-nitrophenyl chloroformate (10 eq each), and DIEA (10 eq each) were stirred in dry dioxane for 3 h at room temperature under nitrogen atmosphere. After addition of acetic acid (25 eq) the mixtures were diluted with 7/3 (v/v) acetonitrile/H<sub>2</sub>O and the carbonates 7a, 7b, 7c, 7d, 7e, 7f, or 7g, respectively, were purified by RP-HPLC.

25

7a MS [M+Na]<sup>+</sup> = 799 (MW+Na calculated = 799.5 g/mol)

7b MS [M+Na]<sup>+</sup> = 785 (MW+Na calculated = 785.5 g/mol)

7c MS [M+Na]<sup>+</sup> = 803 (MW+Na calculated = 803.5 g/mol)

30 7d MS [M+Na]<sup>+</sup> = 1093 (MW+Na calculated = 1093.7 g/mol)

7e MS [M+Na]<sup>+</sup> = 1029 (MW+Na calculated = 1029.6 g/mol)

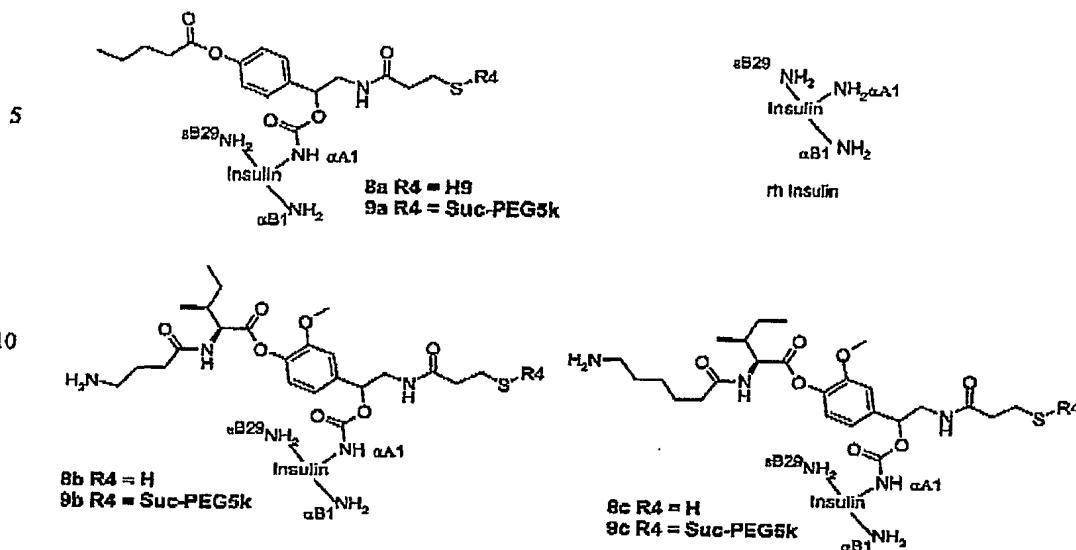
7f MS [M+Na]<sup>+</sup> = 1057 (MW+Na calculated = 1057.6 g/mol)

7g MS [M+Na]<sup>+</sup> = 1026.7 (MW+Na calculated = 1027.6 g/mol)

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# Synthesis of compounds 8a to 8c (N<sup>αA1</sup>-linker-insulin)



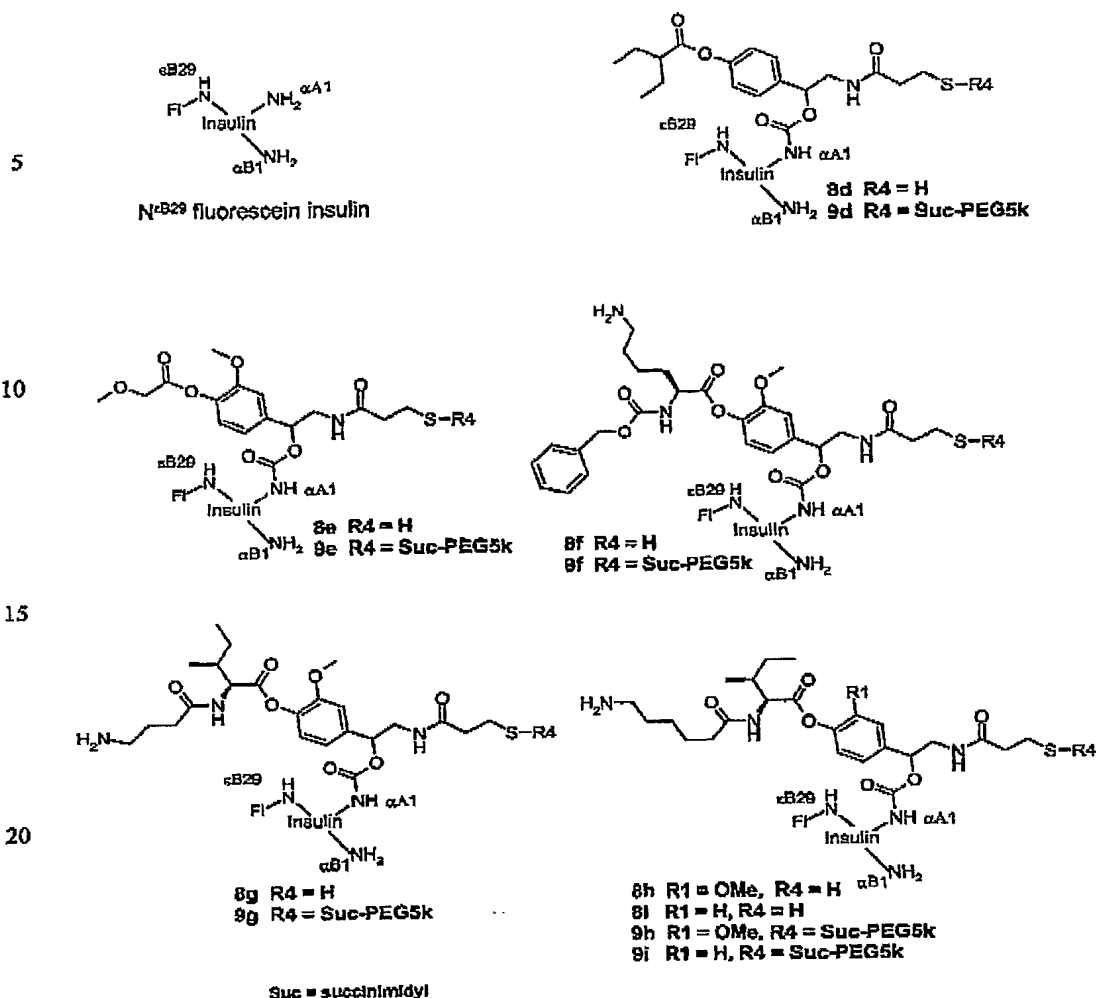
## General synthesis protocol:

- Rh-Insulin in 1/1 (v/v) DMSO/DMF was mixed with a solution of 0.9 eq carbonate 7b, 7e, or 7f in DMSO. The resulting solutions were adjusted to basic pH with DIEA and stirred for 1.5 h at RT. RP-HPLC purification gave Mmt-protected intermediates.
- 20 After lyophilization, the Mmt-protected intermediates were mixed with 95:5 (v/v) TFA/triethylsilane and stirred for 5 min. Volatiles were removed under nitrogen flow and 8a, 8b or 8c, respectively, were purified by RP-HPLC and lyophilized. Position of insulin modification was verified by DTT reduction and MS analysis.
- 25 8a MS  $[M+2H]^{2+} = 3078.9$ ;  $[M+3H]^{3+} = 2053.2$   $[M+4H]^{4+} = 1540.6$  (MW calculated = 6158 g/mol)
- 8b MS  $[M+2H]^{2+} = 3152.9$ ;  $[M+3H]^{3+} = 2100.6$   $[M+4H]^{4+} = 1575.8$  (MW calculated = 6302 g/mol)
- 8c MS:  $[M+3H]^{3+} = 2110.7$ ;  $[M+4H]^{4+} = 1583.7$ ;  $[M+5H]^{5+} = 1266.6$  (MW
- 30 calculated = 6330 g/mol)

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Synthesis of compounds 8d to 8i (N<sup>εB29</sup>-Fluorescein-N<sup>αA1</sup>-linker-insulin)



25 **General synthesis protocol:**

Synthesis of N<sup>εB29</sup>-fluorescein insulin

80 mg (13.8 μmol) rh-insulin were dissolved in 4 ml 1/1 (v/v) DMF/DMSO and 40 μl DIEA were added. 8 mg (17 μmol) 5-(and-6)-carboxyfluorescein succinimidyl ester were added and the solution was stirred for 30 min at room temperature. 4 ml 5/5/1 (v/v/v) acetonitrile/water/acetic acid were added, product N<sup>εB29</sup>-fluorescein insulin was purified by RP-HPLC and lyophilized. The conjugation site was verified by reduction of N<sup>εB29</sup>-fluorescein insulin with 1,4-dithiothreitol, protease digestion and MS analysis.

30

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MS:  $[M+2H]^{2+} = 3084.0$ ;  $[M+3H]^{3+} = 2054.6$  (MW calculated = 6166 g/mol)

General synthesis protocol:

$N^{\text{C}629}$ -fluorescein insulin in 1/1 (v/v) DMF/DMSO was mixed with a solution of 0.9  
5 eq carbonate **7a**, **7c**, **7d**, **7e**, **7f**, or **7g** in DMSO. The resulting solutions were adjusted  
to basic pH with DIEA and stirred for 3 h at RT. RP-HPLC purification gave Mmt-  
protected intermediates.

After lyophilization, the intermediates were dissolved in 95/5 (v/v) TFA/triethylsilane  
and stirred for 5 min. Volatiles were removed under nitrogen flow and **8d**, **8e**, **8f**, **8g**,  
10 **8h** or **8i**, respectively, were purified by RP-HPLC and lyophilized.

**8d** MS:  $[M+2H]^{2+} = 3266.5$ ;  $[M+3H]^{3+} = 2177.8$ ;  $[M+4H]^{4+} = 1633.7$  (MW  
calculated = 6530 g/mol)

**8e** MS:  $[M+3H]^{3+} = 2179.3$ ;  $[M+4H]^{4+} = 1633.9$  (MW calculated = 6534 g/mol)

15 **8f** MS:  $[M+2H]^{2+} = 3364.1$ ;  $[M+3H]^{3+} = 2242.7$ ;  $[M+4H]^{4+} = 1681.5$  (MW  
calculated = 6724 g/mol)

**8g** MS:  $[M+3H]^{3+} = 2219.2$   $[M+4H]^{4+} = 1665.9$ ;  $[M+5H]^{5+} = 1332.8$  (MW  
calculated = 6660 g/mol)

**8h** MS:  $[M+3H]^{3+} = 2229.7$   $[M+4H]^{4+} = 1673.3$ ;  $[M+5H]^{5+} = 1337.7$  (MW calculated  
20 = 6689 g/mol)

**8i** MS:  $[M+3H]^{3+} = 2218.7$   $[M+4H]^{4+} = 1664.9$  (MW calculated = 6659 g/mol)

#### Synthesis of compounds **9a** to **9i** (mono-pegylated insulin compounds)

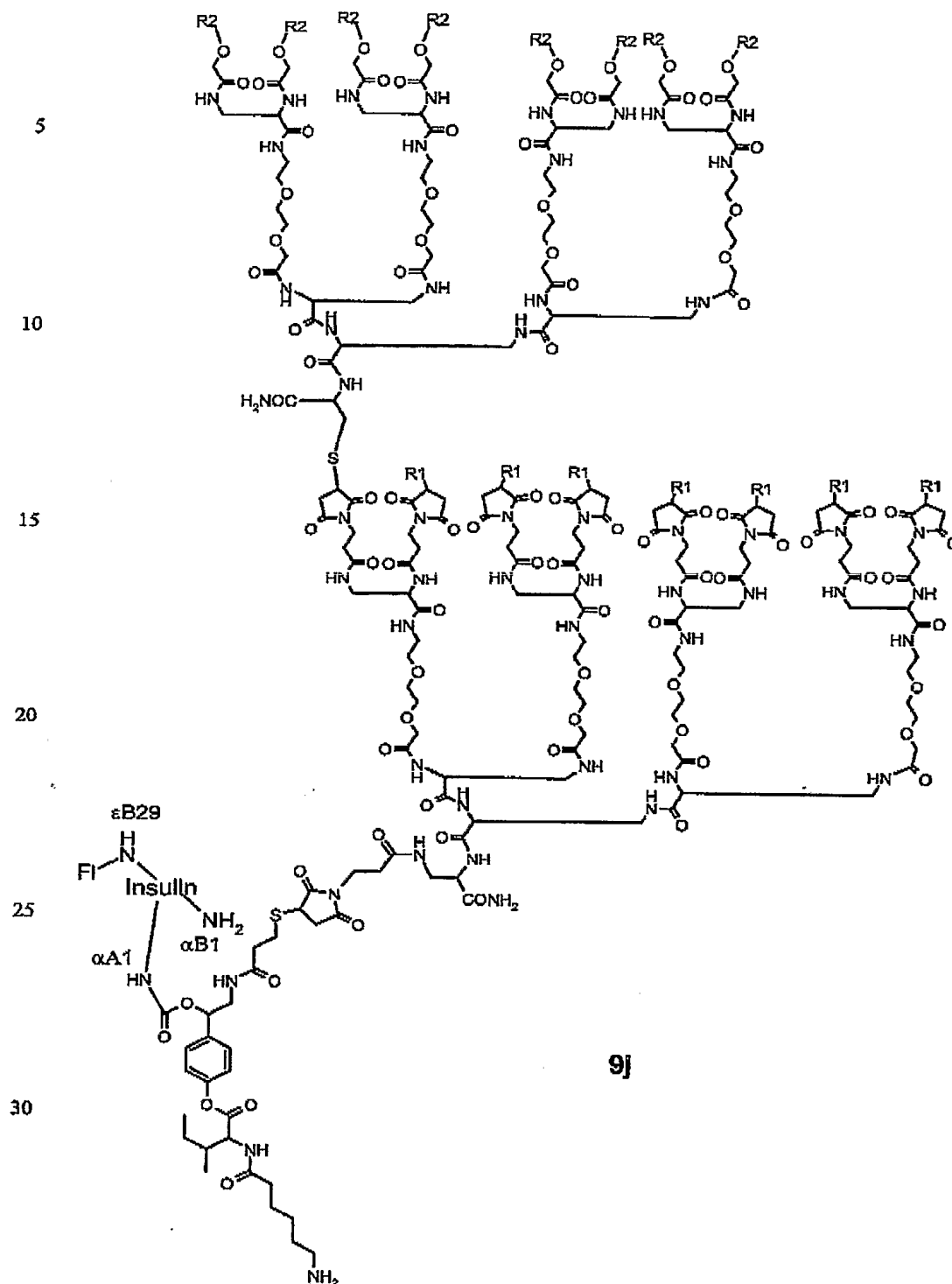
General synthesis protocol:

25 70  $\mu$ l of a solution of **8a**, **8b**, **8c**, **8d**, **8e**, **8f**, **8g**, **8h** or **8i** (500  $\mu$ M) in 1/4 (v/v)  
acetonitrile/water were mixed with 7  $\mu$ l of a solution of 10 mM maleimide-PEG5k  
each in 1/4 (v/v) acetonitrile/water and 10  $\mu$ l of 0.5 M phosphate buffer (pH 7.0) and  
incubated for 15 min. Compounds **9a**, **9b**, **9c**, **9d**, **9e**, **9f**, **9g**, **9h** or **9i**, respectively,  
were purified by SEC (column: Superdex 200, flow rate: 0.75 mL/min) using 10 mM  
30 HEPES buffer (pH 7.4), 150 mM NaCl, 3 mM EDTA, and 0.005 % Tween as mobile  
phase. The collected eluates (approximately 1.5 mL) were directly used for release  
rate determination.

**9a** through **9i**: SEC retention time: 19.5 min

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Synthesis of compound 9j

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5

R2 =

10

15

R1 =

20

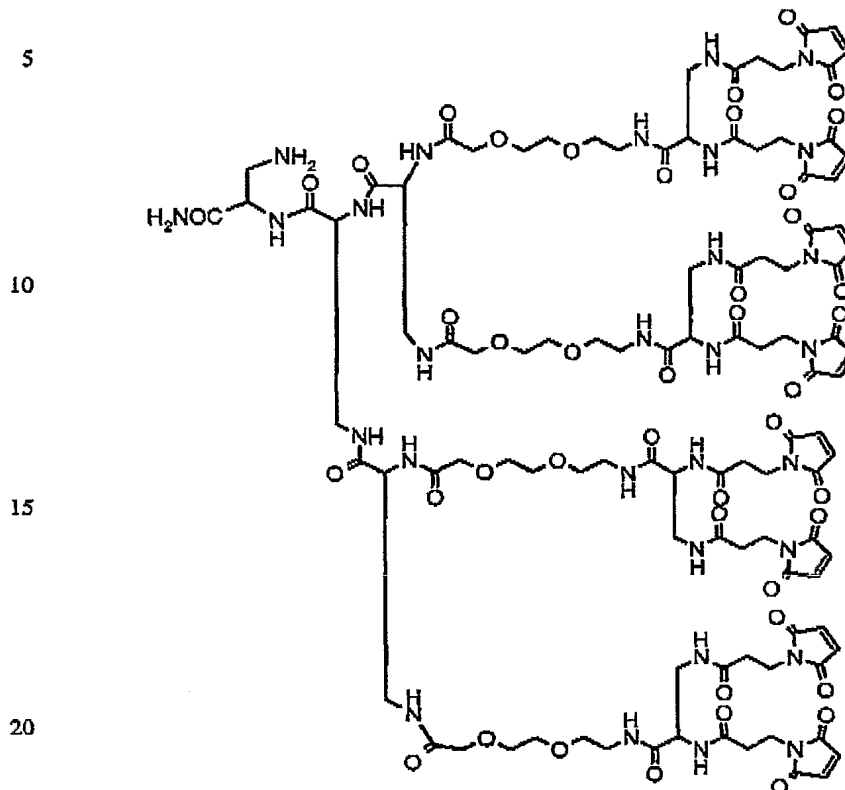
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## Synthesis of compound 10



## 10

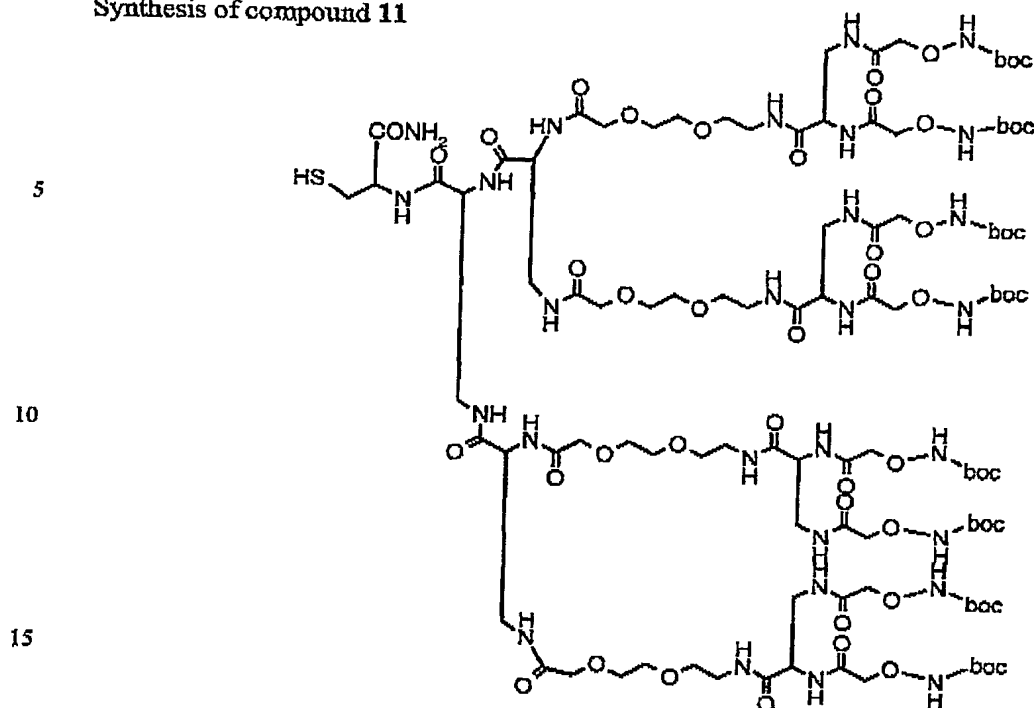
25 10 was obtained according to the standard solid-phase synthesis protocol. The amino acids Fmoc-Dpr(Boc)-OH, Fmoc-Dpr(Fmoc)-OH, Fmoc-Dpr(Fmoc)-OH, Fmoc-Ado-OH, and Fmoc-Dpr(Fmoc)-OH were coupled to TentaGel Sieber amide resin. After final fmoc removal the resin was agitated with 5 eq maleimidopropionic acid and 5 eq DIC in relation to amino groups in DMF for 30 min. 10 was cleaved from  
30 resin with TFA/TES/water 95/3/2 (v/v/v). After evaporation of solvent, product 10 was purified by RP-HPLC.

MS:  $[M+H]^+ = 2494.6$  (MW calculated = 2495.4 g/mol)

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# Synthesis of compound 11



11

Compound 11 was obtained according to the standard solid-phase synthesis protocol.

20 The amino acids Fmoc-Cys(Mmt)-OH, Fmoc-Dpr(Fmoc)-OH, Fmoc-Dpr(Fmoc)-OH, Fmoc-Ado-OH, and Fmoc-Dpr(Fmoc)-OH were coupled to Tentagel Sieber amide resin.

After final fmoc removal the resin was agitated with 3 eq Boc-aminooxyacetic acid, 3 eq DIC, and 3 eq HOBt in relation to amino groups in DMF for 30 min. 11 was  
25 cleaved from resin with DCM/TFA/TES 97/1/2 (v/v/v). After addition of 0.8 eq pyridin in relation to TFA the solvent was evaporated and product 11 was purified by RP-HPLC.

MS:  $[M+H]^+ = 2688.2 \text{ g/mol}$  (MW calculated = 2688.8 g/mol)

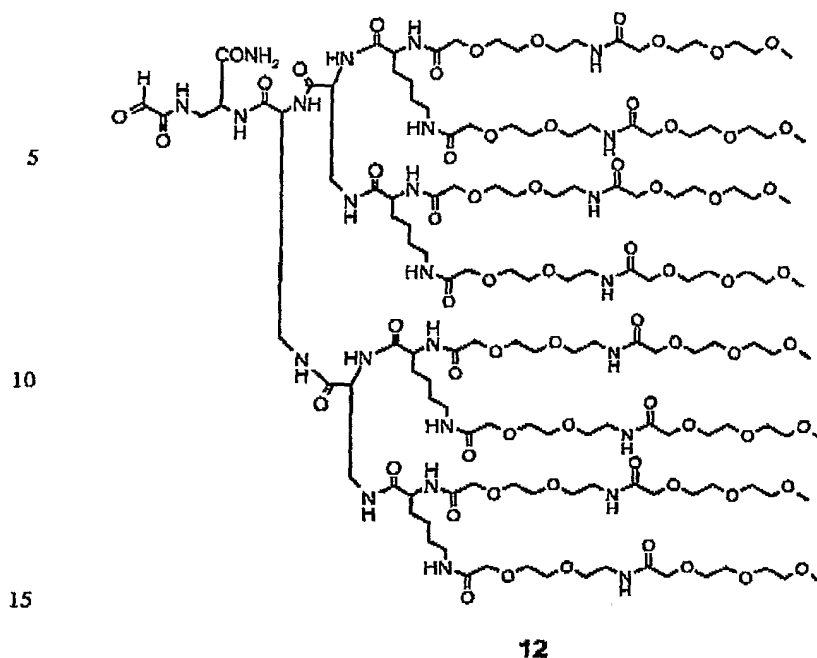
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### Synthesis of compound 12



Compound 12 was obtained according to the standard solid-phase synthesis protocol. The amino acids Fmoc-Dpr(ivDde)-OH, Fmoc-Dpr(Fmoc)-OH, Fmoc-Dpr(Fmoc)-OH, Fmoc-Lys(Fmoc)-OH, and Fmoc-Ado-OH were coupled to TentaGel Sieber  
20 amide resin.

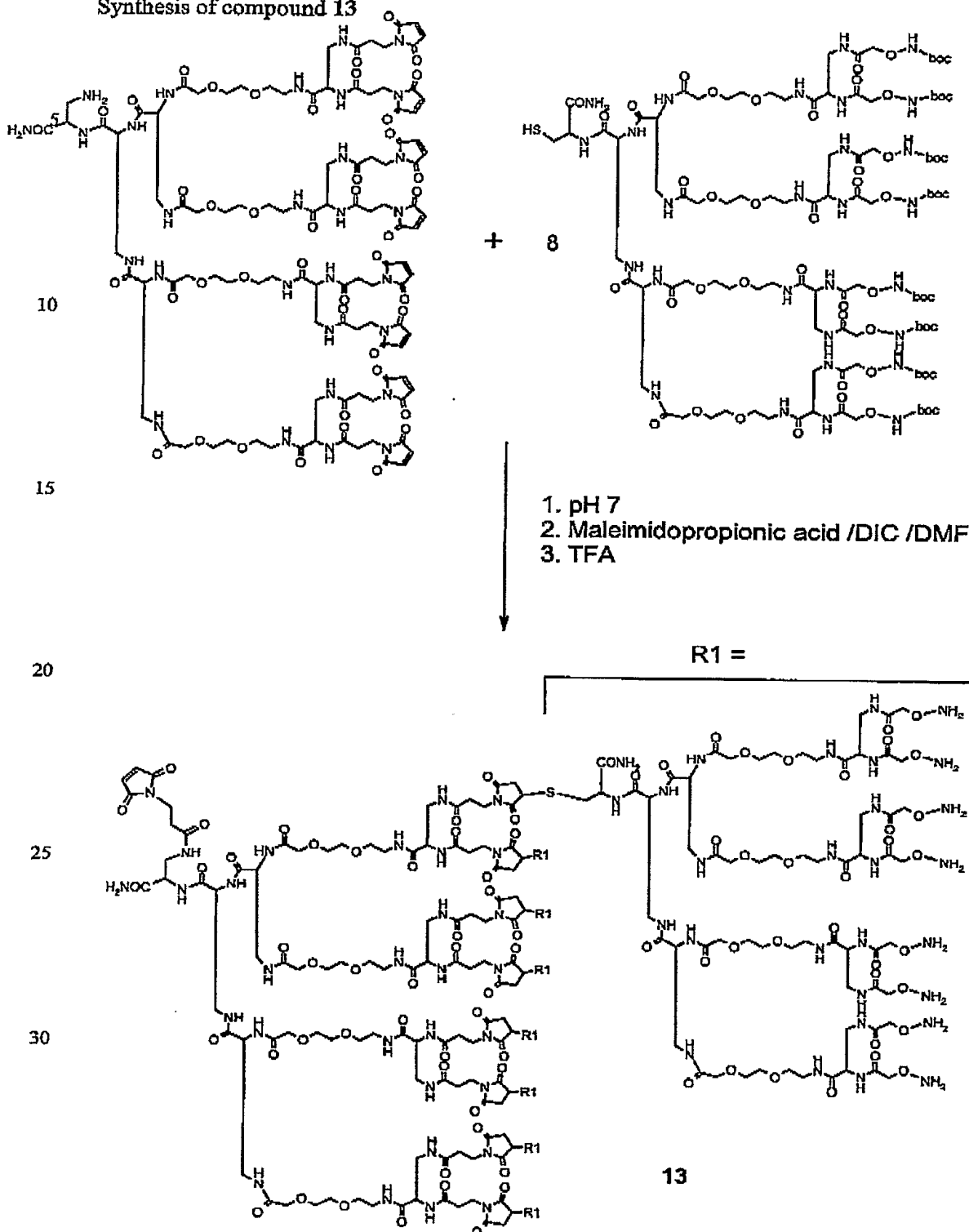
After final fmoc removal the resin was agitated with 3 eq 3,6,9-trioxadecansäure, 3 eq PyBop, and 6 eq DIEA in relation to amino groups in DMF for 60 min.

To cleave the ivDde protecting group, the resin was treated three times with 2 %  
25 Hydrazin in DMF. After washing, 3 eq Fmoc-Ser-OH was coupled with 3eq DIC and 3 eq HOBt for 30 min. After final fmoc removal resin was washed and the product was cleaved from resin with DCM/TFA/TES 88/10/2 (v/v/v). Solvent was evaporated and the residue was oxidized with 10 eq sodium periodate in 3/2 (v/v) 0.1 M sodium phosphate pH 7 / acetonitrile for 15 min to yield 12. Product 12 was purified by RP-  
30 HPLC and lyophilized.

MS:  $[M+H]^+ = 3372.1 \text{ g/mol}$  (3372.8 g/mol)

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### Synthesis of compound 13



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6 mg (2.4  $\mu$ mol) of compound 10 were dissolved in 1 ml 2/1 (v/v) acetonitrile / 0.1 M sodium phosphate buffer pH 7 and 65 mg (24.2  $\mu$ mol) of compound 11 were added. The solution was stirred at room temperature for 2 h and then the product purified by  
5 RP-HPLC and lyophilized (yield: 45 mg (78 %)).

The lyophilized product (45 mg) was dissolved in 0.5 ml DMF and 10  $\mu$ l DIEA were added. 5 mg (30  $\mu$ mol) 3-maleimidopropionic acid and 4.7  $\mu$ l (30  $\mu$ mol) DIC in 150  $\mu$ l DMF were added and the reaction mixture was stirred at room temperature for 20  
10 min, the product purified by RP-HPLC and lyophilized.

The lyophilized product was incubated for 10 min in 95/5 (v/v) TFA/water and then the solvent was removed in a stream of nitrogen. Product 13 was purified by RP-HPLC and lyophilized (overall yield for all three steps: 20 mg (47 %)).

15

MS: 17700 – 18200 (broad peak) (MW calculated = 17749 g/mol)

#### Synthesis of compound 9j

20

1.5 mg (225 nmol) 8i and 5 mg (280 nmol) 13 were mixed, dissolved in 300  $\mu$ l 2/1 (v/v) 0.1 M sodium phosphate buffer pH 7 / acetonitrile and incubated for 15 min at room temperature. The product was purified by RP-HPLC and lyophilized. (yield 4 mg (160 nmol, 70 %)

25

The lyophilized product was dissolved in 200  $\mu$ l 0.1 M sodium citrate buffer pH 1.5 and 69 mg (20.5  $\mu$ mol) 12 in 200  $\mu$ l 2/1 (v/v) acetonitrile / sodium citrate buffer pH 1.5 were added. The mixture was stirred at room temperature for 24 h and product 9j was purified by size exclusion chromatography (column: Superdex 200, buffer: 10  
30 mM HEPES pH 7.4, 0.005 % Tween-20, 3mM EDTA, flow rate: 0.75 ml/min)

SEC elution time: 15 min

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Release of insulin or fluorescein-insulin from conjugates in buffer pH 7.4

5 Release of (fluorescein)-insulin from (fluorescein)-insulin conjugate was effected by  
linker hydrolysis in aqueous buffer pH 7.4. Collected SEC eluates of (fluorescein)  
insulin conjugates (see above) were incubated at 37 °C and samples were taken at  
time intervals and analyzed by RP-HPLC (insulin conjugates) or SEC (fluorescein  
insulin conjugates) and UV detection. Peaks correlating with the retention time of  
10 native insulin or fluorescein-insulin, respectively, were integrated and plotted against  
incubation time, and curve-fitting software was applied to estimate the corresponding  
halftime of release.

Release of fluorescein-insulin from conjugate 9e to 9g in 80 % human plasma

15 Release of fluorescein-insulin from 9e, 9f or 9g was effected by hydrolysis in 80 %  
human plasma in 20 mM HEPES pH 7.4 at 37 °C. Samples were taken at time  
intervals and analyzed by SEC and UV detection. Peaks correlating with the retention  
time of fluorescein-insulin were integrated and plotted against incubation time, and  
curve-fitting software was applied to estimate the corresponding halftime of release.

20

Table: Polymeric prodrug hydrolysis

compound	t <sub>1/2</sub> buffer pH 7.4	t <sub>1/2</sub> human plasma (80%)
9a	40 h	nd
9b	55 h	nd
9c	4.5 d	nd
9d	> 7 d	nd
9e	11.5 h	6 h
9f	7 h	4 h
9g	55 h	30 h
9h	90 h	nd
9i	37 h	nd
9j	88 h	nd

nd = not determined

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Abbreviations:

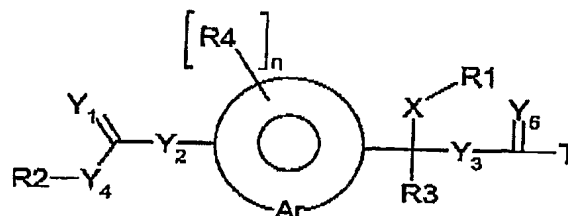
	Boc	t-butyloxycarbonyl
	DBU	1,3-diazabicyclo[5.4.0]undecene
	DCM	dichloromethane
5	(iv)Dde	1-(4,4-dimethyl-2,6-dioxo-cyclohexyliden)3-methyl-butyl
	DIC	diisopropylcarbodiimide
	DIEA	diisopropylethylamine
	DMAP	dimethylamino-pyridine
	DMF	N,N-dimethylformamide
10	DMSO	dimethylsulfoxide
	Dpr	diaminopropionic acid
	EDTA	ethylenediaminetetraacetic acid
	eq	stoichiometric equivalent
	fmoc	9-fluorenylmethoxycarbonyl
15	Fmoc-Ado-OH	Fmoc-8-amino-3,6-dioxaoctanoic acid
	HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)
	HOBT	N-hydroxybenzotriazole
	LCMS	mass spectrometry-coupled liquid chromatography
	Mal	maleimidopropionyl
20	Mmt	4-methoxytrityl
	MS	mass spectrum
	MW	molecular mass
	PyBop	benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
25	RP-HPLC	reversed-phase high performance liquid chromatography
	RT	room temperature
	SEC	size exclusion chromatography
	Suc	succinimidopropionyl
	TES	triethylsilane
30	TFA	trifluoroacetic acid
	THF	tetrahydrofuran
	UV	ultraviolet
	Z	benzyloxycarbonyl

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## CLAIMS

1. A molecule having the following structure;



10 wherein

T is D or A

D being a residue of an amine containing biologically active molecule and  
 A being a leaving group;

X is a spacer moiety such as R5-Y6

15 Y<sub>1</sub>, Y<sub>2</sub> can each be either O, S, or NR<sub>6</sub>, independently of each other.

Y<sub>3</sub>, Y<sub>5</sub> can each be either O or S, independently of each other.

Y<sub>4</sub> is O, NR<sub>6</sub>, or -C(R<sub>7</sub>)(R<sub>8</sub>)-

Y<sub>6</sub> is O, S, NR<sub>6</sub>, succinimide, maleimide, unsaturated carbon-carbon bonds or  
 any heteratom containing a free electron pair.

20 R<sub>3</sub> is selected from hydrogen, substituted or non-substituted linear, branched  
 or cyclical alkyl or heteroalkyl, aryls, substituted aryls, substituted or non-  
 substituted heteroaryl, cyano, nitro, halogen, carboxy, carboxyalkyl,  
 alkylcarbonyl, or carboxamidoalkyl;

25 R<sub>4</sub> is selected independently from hydrogen, substituted or non-substituted  
 linear, branched or cyclical alkyl or heteroalkyl, aryl, substituted aryl,  
 substituted or non-substituted heteroaryl, substituted or non-substituted linear,  
 branched, or cyclical alkoxy, substituted or non-substituted linear, branched,  
 or cyclical heteroalkyloxy, aryloxy, or heteroaryloxy;

30 R<sub>5</sub> is selected from substituted or non-substituted linear, branched or cyclical  
 alkyl or heteroalkyl, aryls, substituted aryls, substituted or non-substituted  
 heteroaryl, carboxyalkyl, alkylcarbonyl or carboxamidoalkyl;

R<sub>7</sub> and R<sub>8</sub> are selected from hydrogen, substituted or non-substituted linear,  
 branched or cyclical alkyl or heteroalkyl, aryls, substituted aryls, substituted or

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non-substituted heteroaryls, carboxyalkyl, alkylcarbonyl, carboxamidoalkyl,  
cyano, or halogen;  
R6 is selected from hydrogen, substituted or non-substituted linear, branched  
or cyclical alkyl or heteroalkyl, aryls, substituted aryls, substituted or non-  
substituted heteroaryls;  
5 R1 is a polymer;  
R2 is selected from hydrogen, substituted or non-substituted linear alkyl,  
branched alkyl or cycloalkyl, aryl, substituted aryl, substituted or non-  
substituted linear heteroalkyl, branched heteroalkyl or cyclical heteroalkyl,  
10 substituted or nonsubstituted heteroaryl, substituted or non-substituted linear  
alkoxy, branched alkoxy, or cyclical alkoxy, substituted or non-substituted  
linear heteroalkyloxy, branched heteroalkyloxy, or cyclical heteroalkyloxy,  
aryloxy, heteroaryloxycyano, carboxy, carboxyalkyl, alkylcarbonyl,  
carboxamidoalkyl, peptide sequences which can be cleaved by specific  
15 enzymes;  
n is zero or a positive integer; and  
Ar is a multi-substituted aromatic hydrocarbon or a multi-substituted aromatic  
heterocycle.

- 20 2. The molecule of claim 1, wherein the biologically active molecule is selected  
from the group of biologically molecules consisting of small molecule  
biologically active agents or biopolymers.
- 25 3. The molecule of claim 2, wherein the biopolymers are selected from the group  
of biopolymers consisting of proteins, polypeptides, oligonucleotides and  
peptide nucleic acids.
- 30 4. The molecule of claim 3, wherein the polypeptides are selected from the group  
of polypeptides consisting of ACTH, adenosine deaminase, agalsidase,  
albumin, alfa-1 antitrypsin (AAT), alfa-1 proteinase inhibitor (API), alteplase,  
anistreplase, anicrod serine protease, antibodies (monoclonal or polyclonal, and  
fragments or fusions), antithrombin III, antitrypsins, aprotinin, asparaginases,  
biphalin, bone-morphogenic proteins, calcitonin (salmon), collagenase,  
DNase, endorphins, enfuvirtide, enkephalins, erythropoietins, factor VIIa,

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- factor VIII, factor VIIIa, factor IX, fibrinolysin, fusion proteins, follicle-stimulating hormones, granulocyte colony stimulating factor (G-CSF), galactosidase, glucagon, glucocerebrosidase, granulocyte macrophage colony stimulating factor (GM-CSF), phospholipase-activating protein (PLAP),
- 5 gonadotropin chorionic (hCG), hemoglobins, hepatitis B vaccines, hirudin, hyaluronidases, iduronidase, immune globulins, influenza vaccines, interleukins (1 alfa, 1 beta, 2, 3, 4, 6, 10, 11, 12), IL-1 receptor antagonist (rhIL-1ra), insulins, interferons (alfa 2a, alfa 2b, alfa 2c, beta 1a, beta 1b, gamma 1a, gamma 1b), keratinocyte growth factor (KGF), transforming
- 10 growth factors, lactase, leuprolide, levothyroxine, luteinizing hormone, Lyme vaccine, natriuretic peptide, pancrelipase, papain, parathyroid hormone, PDGF, pepsin, platelet activating factor acetylhydrolase (PAF-AH), prolactin, protein C, octreotide, secretin, sermorelin, superoxide dismutase (SOD), somatropins (growth hormone), somatostatin, streptokinase, sucrase, tetanus
- 15 toxin fragment, tilactase, thrombins, thymosin, thyroid stimulating hormone, thyrotropin, tumor necrosis factor (TNF), TNF receptor-IgG Fc, tissue plasminogen activator (tPA), TSH, urate oxidase, urokinase, vaccines, and plant protein such as lectin and ricin.
- 20 5. The molecule of claim 3, wherein the protein is a protein prepared by recombinant DNA technology.
- 25 6. The molecule of claim 3, wherein the protein is selected from the group of proteins consisting of antibody fragments, single chain binding proteins, catalytic antibodies and fusion proteins.
- 30 7. The molecule of claim 2, wherein the small molecule biologically active agents are selected from the group of agents consisting of central nervous system-active agents, anti-infective, anti-neoplastic, antibacterial, anti-fungal, analgesic, contraceptive, anti-inflammatory, steroidal, vasodilating, vasoconstricting, and cardiovascular agents with at least one primary or secondary amino group.



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8. The molecule of claim 2, wherein the small molecule biologically active agents are selected from the group of compounds consisting of daunorubicin, doxorubicin, idarubicin, mitoxantron, aminoglutethimide, amantadine, diaphenylsulfon, ethambutol, sulfadiazin, sulfamerazin, sulfamethoxazol, sulfalen, clinafloxacin, moxifloxacin, ciprofloxacin, enoxacin, norfloxacin, neomycin B, spectinomycin, kanamycin A, meropenem, dopamin, dobutamin, lisinopril, serotonin, acivicin and carbutamid.
9. The molecule of claim 1, wherein R4 is selected from the group of small substituents consisting of hydrogen, methyl, ethyl, ethoxy, methoxy, and other linear alkyls, cycloalkyls or branched alkyls and heteroalkyl comprising one to six carbon atoms.
10. The molecule of claim 1, wherein R1 is selected from the group of polymers consisting of polyalkyloxy-based polymers like poly(propylene glycol) or poly(ethylene glycol), dextran, chitosan, hyaluronic acid and derivatives, alginate, agarose, cellulose, hydroxyethyl starch (HES) and other carbohydrate-based polymers, poly(vinyl alcohols), poly(oxazolines), poly(anhydrides), poly(ortho esters), poly(carbonates), poly(urethanes), poly(acrylic acids), poly(acrylamides) such as hydroxypropylmethacrylamide (HMPA), poly(organophosphazenes), poly(vinylpyrrolidone), poly(cyanoacrylates), poly(esters) such as poly(lactic acid) or poly(glycolic acids), poly(iminocarbonates), poly(amino acids) such as poly(glutamic acid), collagen, gelatin, copolymers, grafted copolymers, cross-linked polymers, and block copolymers from the above listed polymers.
11. The molecule of claim 1, wherein R1 is a branched or hyperbranched polymer.
12. The molecule of claim 1, wherein R1 is a dendrimer or dense star polymer.
13. The molecule of claim 1, wherein R1 is a biopolymer.
14. The molecule of claim 13, wherein R1 is a protein.

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15. The molecule of claim 14, wherein the protein is albumin, an antibody, fibrin, casein or any other plasma protein.
- 5 16. The molecule of any one of the above claims, wherein R1 further includes one or more biologically active substances.
17. The molecule of any one of the above claims, wherein the polymer of R1 has at least one functional group for linkage to X.
- 10 18. The molecule of claim 17, wherein the at least one functional group is selected from the group of functional groups consisting of carboxylic acid and activated derivatives, amino, maleimide, thiol, sulfonic acid and derivatives, carbonate and derivatives, carbamate and derivatives, hydroxyl, aldehyde, ketone, hydrazine, isocyanate, isothiocyanate, phosphoric acid and derivatives, phosphonic acid and derivatives, haloacetyl, alkyl halides, 15 acryloyl, arylating agents like aryl fluorides, hydroxylamine, disulfides like pyridyl disulfide, vinyl sulfone, vinyl ketone, diazoalkanes, diazoacetyl compounds, epoxide, oxirane, and aziridine.
- 20 19. The molecule of claim 17 or 18, wherein the at least one functional group is selected from the group of functional groups consisting of thiol, maleimide, amino, carboxylic acid and derivatives, carbonate and derivatives, carbamate and derivatives, aldehyde, and haloacetyl.
- 25 20. The molecule of one of claims 17 to 19, wherein the at least one functional group is selected from the group of functional groups consisting of thiol, maleimide, amino, carboxylic acid and derivatives, carbamate and derivatives, and carbonate and derivatives thereof.
- 30 21. The molecule of any one of the above claims, wherein the polymer is a cross-linked polymer.
22. The molecule of any one of the above claims, wherein A is selected from the group of leaving groups consisting of chloride, nitrophenoxy, imidazolyl, N-

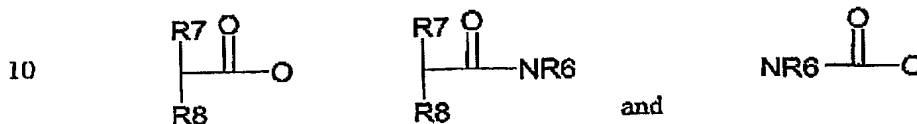
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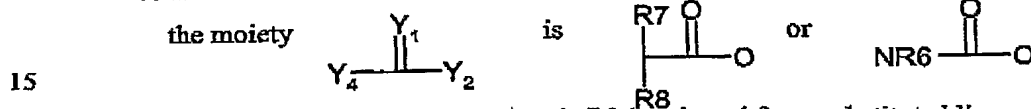
hydroxysuccinimidyl, N-hydroxybenzotriazolyl, N-hydroxyazobenzotriazolyl, pentafluorophenoxy and N-hydroxysulfosuccinimidyl.

23. The molecule of claim 1, wherein R2 is selected from non-toxic substituted or non-substituted linear alkyl, branched alkyl or cyclical alkyl or heteroalkyl.

24. The molecule of claim 1, wherein the moiety  $Y_4 - \overset{Y_1}{\underset{\text{||}}{C}} - Y_2$  is preferably selected from the group of moieties consisting of



25. The molecule of claim 24, wherein



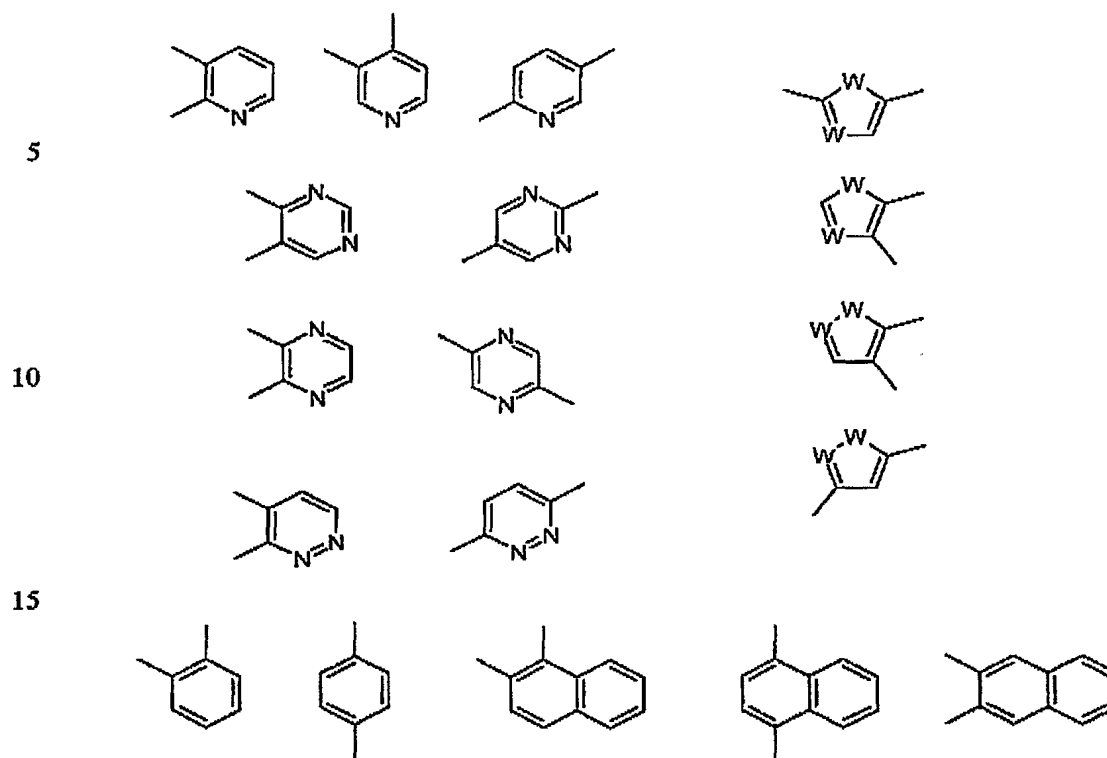
26. The molecule of claim 25, wherein R2 is selected from substituted linear, branched or cyclical alkyl or heteroalkyl, containing preferably a nucleophile that can perform a nucleophilic attack at the carbonyl carbon and thus catalyse the cleavage of a masking group by intramolecular catalysis or cyclization

27. The molecule of claim 25 or 26, wherein R7 and/or R8 are not hydrogen.

28. The molecule of claim 1, wherein Ar is selected from

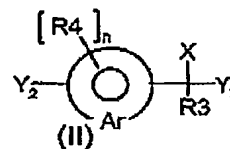
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and wherein W is O, S, or N.

29. Use of the molecule of any one of claims 1 to 28, in which R1 is a protecting group PG1 and T is a leaving group A, for covalent conjugation with a biologically active molecule and linkage with a carrier.
30. The use of claim 29, wherein the biologically active molecule is an amine-containing molecule.
31. The use of claim 29 or 30, wherein the biologically active molecule is a peptide, polypeptide or protein.
32. Method for the synthesis of a molecule comprising:
- providing a starting molecule of Formula II



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- synthesising at least one intermediate compound from the starting molecule of Formula II; and  
- attaching an amine-containing biologically active molecule to the at least one intermediate compound;

5

wherein

$Y_2$  is selected from O, S, or NR6

$Y_3$  is selected from O or S

X is a spacer moiety such as R5-Y6;

10

R3 is selected from hydrogen, substituted or non-substituted linear, branched or cyclical alkyl or heteroalkyl, aryl, substituted aryl, substituted or non-substituted heteroaryl, cyano, nitro, halogen, carboxy, carboxyalkyl, alkylcarbonyl or carboxamidoalkyl;

15

R4 is selected from hydrogen, substituted or non-substituted linear, branched or cyclical alkyl or heteroalkyl, aryl, substituted aryl, substituted or non-substituted heteroaryl, substituted or non-substituted linear, branched, or cyclical alkoxy, substituted or non-substituted linear, branched, or cyclical heteroalkyloxy, aryloxy or heteroaryloxy;

20

R5 is selected from substituted or non-substituted linear, branched or cyclical alkyl or heteroalkyl, aryl, substituted aryl, substituted or non-substituted heteroaryl, carboxyalkyl, alkylcarbonyl, carboxamidoalkyl;

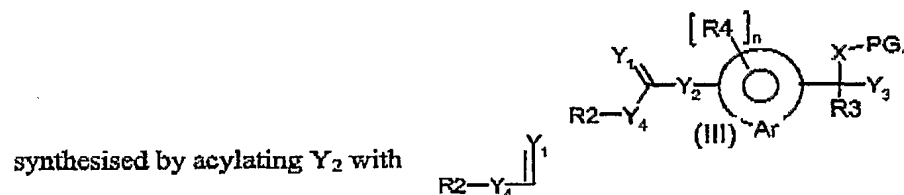
R6 is selected from hydrogen, substituted or non-substituted linear, branched or cyclical alkyl or heteroalkyl, aryl, substituted aryl, substituted or non-substituted heteroaryl and

25

$Y_6$  is O, S, NR6, succinimide, maleimide, unsaturated carbon-carbon bonds or a heteratom containing a free electron pair.

33. The method of claim 32, wherein a first one of the at least one intermediate compounds is an intermediate molecule of Formula III

30



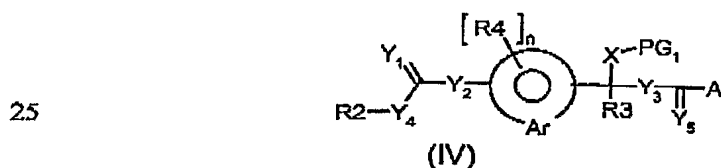
wherein R2 is selected from hydrogen, substituted or non-substituted linear

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- alkyl, branched alkyl or cycloalkyl, aryl, substituted aryl, substituted or non-substituted linear, branched or cyclical heteroalkyl, substituted or nonsubstituted heteroaryl, substituted or non-substituted linear alkoxy, branched alkoxy, or cyclical alkoxy, substituted or non-substituted linear
- 5 heteroalkyloxy, branched heteroalkyloxy, or cyclical heteroalkyloxy, aryloxy, heteroaryloxy, cyano, carboxy, carboxyalkyl, alkylcarbonyl, carboxamidoalkyl, or peptide sequences which can be cleaved by specific enzymes;
- $Y_1$  is selected from the group of moieties consisting of O, S, or NR6;
- $Y_4$  is selected from the group of moieties consisting of, or  $-C(R7)(R8)-O$ ,
- 10 NR6;
- $R6$  is selected from hydrogen, substituted or non-substituted linear, branched or cyclical alkyl or heteroalkyl, aryl, substituted aryl, substituted or non-substituted heteroaryl;
- $R7$  and  $R8$  are selected from hydrogen, substituted or non-substituted linear,
- 15 branched or cyclical alkyl or heteroalkyl, aryl, substituted aryl, substituted or non-substituted heteroaryl, carboxyalkyl, alkylcarbonyl, carboxamidoalkyl, cyano or halogen.
- $PG_1$  is a first protecting group or absent.

- 20 34. The method of claim 33, wherein a second one of the at least one intermediate compound is an intermediate compound of formula IV



formed by activating the compound of Formula III with an activating agent, wherein A is a leaving group and  $Y_5$  is selected from O or S.

- 30 35. The method of claim 34, wherein A is selected from chloride, bromide, fluoride, nitrophenoxy, imidazolyl, N-hydroxysuccinimidyl, N-hydroxybenzotriazolyl, N-hydroxyazobenzotriazolyl, pentafluorophenoxy and N-hydroxysulfosuccinimidyl.

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36. The method of claim 34 or 35, wherein the activating agent is selected from the group of activating agents consisting of 4-nitrophenyl chloroformate or disuccinyl carbonate.

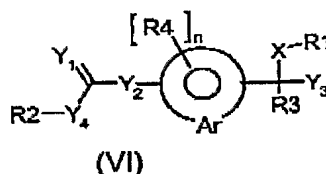
5 37. The method of one of claims 34 or 36, wherein the amine-containing biologically active molecule is attached to the compound of Formula IV by displacement of the leaving group A.

10 38. The method of claim 37 comprising a step of removal of the reversible first protecting group PG<sub>1</sub>.

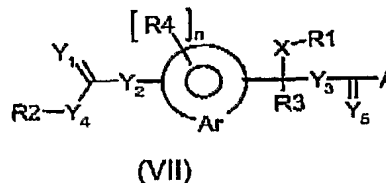
15 39. The method of claim 38, wherein the step of removal of the reversible protection group PG<sub>1</sub> is carried out using a reagent selected from the group of reagents consisting of trifluoroacetic acid or DTT.

40. The method of any one of claims 38 or 39, further comprising a step of attaching a polymer R1 to X.

20 41. The method of claim 33, further comprising a step of attaching a polymer R1 to X to form a third one of the at least one intermediate compound and having a Formula VI.



25 42. The method of claim 41, further comprising a step of activating the third one of the at least one intermediate compound by using an activating agent to form a fourth one of the at least one intermediate compound and having a Formula VII and wherein A is a leaving group



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43. The method of claim 42, wherein A is selected from chloride, bromide, fluoride, nitrophenoxy, imidazolyl, N-hydroxysuccinimidyl, N-hydroxybenzotriazolyl, N-hydroxyazobenzotriazolyl, pentafluorophenoxy and N-hydroxysulfosuccinimidyl.

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44. The method of claim 42 or 43, wherein the activating agent is selected from the group of activating agents consisting of 4-nitrophenyl chloroformate or disuccinyl carbonate.

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45. The method of one of claim 42 to 44, wherein the amine-containing biologically active molecule is attached to the compound of Formula VII by displacement of the leaving group A.

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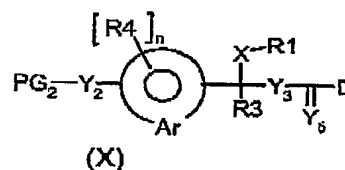
46. The method of claim 32, further comprising a step of reacting the starting molecule of Formula II with a first polymer R1 to attach the first polymer R1 to X.

47. The method of claim 46, further comprising a step of protecting Y<sub>2</sub> with a second protective group PG<sub>2</sub>.

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48. The method of claim 46 or 47, further comprising the step of activating the molecule with an activating agent and reacting with an amine-containing biologically active molecule to form a fifth one of the at least one intermediate compound and having Formula X

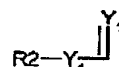
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49. The method of claim 48, wherein the activating agent is selected from the group of activating agents consisting of 4-nitrophenyl chloroformate or disuccinyl carbonate

50. The method of claim 49 or 50, wherein the second protecting group PG<sub>2</sub> is removed from Y<sub>2</sub> and Y<sub>2</sub> is acylated with





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wherein R2 is selected from hydrogen, substituted or non-substituted linear alkyl, branched alkyl or cycloalkyl, aryl, substituted aryl, substituted or non-substituted linear heteroalkyl, branched heteroalkyl or cyclical heteroalkyl, substituted or nonsubstituted heteroaryl, substituted or non-substituted linear alkoxy, branched alkoxy, or cyclical alkoxy, substituted or non-substituted linear heteroalkyloxy, branched heteroalkyloxy, or cyclical heteroalkyloxy, aryloxy, heteroaryloxy, cyano, carboxy, carboxyalkyl, alkylcarbonyl, carboxamidoalkyl, peptide sequences which can be cleaved by specific enzymes;

Y<sub>1</sub> is selected from the group of moieties consisting of O, S, or NR<sub>6</sub>;

Y<sub>4</sub> is selected from the group of moieties consisting of -C(R<sub>7</sub>)(R<sub>8</sub>)-, O, NR<sub>6</sub>;

R<sub>6</sub> is selected from hydrogen, substituted or non-substituted linear, branched or cyclical alkyl or heteroalkyl, aryl, substituted aryl, substituted or non-substituted heteroaryl; and

R<sub>7</sub> and R<sub>8</sub> are selected from hydrogen, substituted or non-substituted linear, branched or cyclical alkyl or heteroalkyl, aryl, substituted aryl, substituted or non-substituted heteroaryl, carboxyalkyl, alkylcarbonyl, carboxamidoalkyl, cyano or halogen.

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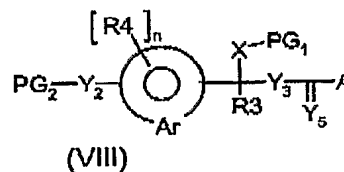
51. The method of claim 50, wherein the removal of the second reversible group

PG<sub>2</sub> is carried out using a reagent selected from the group of reagents consisting of trifluoroacetic acid or DTT.

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52. The method of claim 32 comprising a step of attaching a removable first protecting group PG<sub>1</sub> to X and a removable second protecting group PG<sub>2</sub> to the starting compound of Formula II and thereafter activation using an activating agent to form a sixth one of the at least one intermediate compound and having Formula VIII, wherein A is a leaving group

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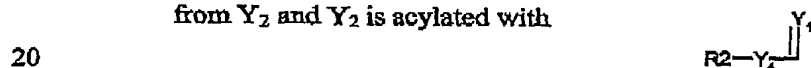
53. The method of claim 53, wherein A is selected from the group of leaving groups selected from the group of leaving groups consisting of chloride, nitrophenoxy, imidazolyly, N-hydroxysuccinimidyl, N-hydroxybenzotriazolyl, N-hydroxyazobenzotriazolyl, pentafluorophenoxy and N-hydroxysulfosuccinimidyl.

54. The method of claim 52 or 53, wherein the activating agent is selected from the group of activating agents consisting of 4-nitrophenyl chloroformate or disuccinyl carbonate.

55. The method of any one of claims 52 or 54, wherein the amine-containing biologically active molecule is attached to the sixth one of the at least one intermediate compound by displacement of the leaving group A.

56. The method of claim 55 comprising a step of removing the first protecting group PG<sub>1</sub> from X and attaching a polymer R<sub>1</sub>.

57. The method of claim 56, wherein the second protecting group PG<sub>2</sub> is removed from Y<sub>2</sub> and Y<sub>2</sub> is acylated with



wherein R<sub>2</sub> is selected from hydrogen, substituted or non-substituted linear alkyl, branched alkyl or cycloalkyl, aryl, substituted aryl, substituted or non-substituted linear heteroalkyl, branched heteroalkyl or cyclical heteroalkyl, substituted or nonsubstituted heteroaryl, substituted or non-substituted linear alkoxy, branched alkoxy, or cyclical alkoxy, substituted or non-substituted linear heteroalkyloxy, branched heteroalkyloxy, or cyclical heteroalkyloxy, aryloxy, heteroaryloxy, cyano, carboxy, carboxyalkyl, alkylcarbonyl, carboxamidoalkyl, peptide sequences which can be cleaved by specific enzymes;

Y<sub>1</sub> is selected from the group of moieties consisting of O, S, or NR<sub>6</sub>;

Y<sub>4</sub> is selected from the group of moieties consisting of -C(R<sub>7</sub>)(R<sub>8</sub>)-; O, or NR<sub>6</sub>;

R<sub>6</sub> is selected from hydrogen, substituted or non-substituted linear, branched or cyclical alkyl or heteroalkyl, aryl, substituted aryl, substituted or non-

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substituted heteroaryl; and

R7 and R8 are selected from hydrogen, substituted or non-substituted linear, branched or cyclical alkyl or heteroalkyl, aryl, substituted aryl, substituted or non-substituted heteroaryl, carboxyalkyl, alkylcarbonyl, carboxamidoalkyl, cyano or halogen.

58. The method of claim 55, wherein the second protecting group PG<sub>2</sub> is removed from Y<sub>2</sub> and Y<sub>2</sub> is acylated with



wherein R2 is selected from hydrogen, substituted or non-substituted linear alkyl, branched alkyl or cycloalkyl, aryl, substituted aryl, substituted or non-substituted linear heteroalkyl, branched heteroalkyl or cyclical heteroalkyl, substituted or nonsubstituted heteroaryl, substituted or non-substituted linear alkoxy, branched alkoxy, or cyclical alkoxy, substituted or non-substituted linear heteroalkyloxy, branched heteroalkyloxy, or cyclical heteroalkyloxy, aryloxy, heteroaryloxy, cyano, carboxy, carboxyalkyl, alkylcarbonyl, carboxamidoalkyl, peptide sequences which can be cleaved by specific enzymes;

Y1 is selected from the group of moieties consisting of O, S, or NR6;

Y4 is selected from the group of moieties consisting of or -C(R7)(R8)- O, or NR6;

R6 is selected from hydrogen, substituted or non-substituted linear, branched or cyclical alkyl or heteroalkyl, aryl, substituted aryl, substituted or non-substituted heteroaryl; and

R7 and R8 are selected from hydrogen, substituted or non-substituted linear alkyl, branched alkyl or cyclical alkyl, aryl, substituted aryl, substituted or non-substituted heteroalkyl or heteroaryl, carboxyalkyl, alkylcarbonyl, carboxamidoalkyl, cyano or halogen.

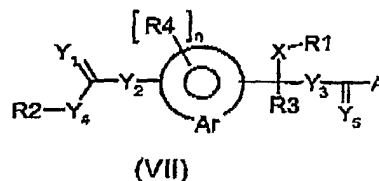
59. The method of claim 58 comprising a step of removing the first protecting group PG<sub>1</sub> from X and attaching a polymer R1.

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60. The method of claim 46 comprising a step of removing the first protecting group  $PG_1$  from X and attaching a polymer R1.

61. The method of claim 60, further comprising a step of activating the third one of the at least one intermediate compound by using an activating agent to form a seventh one of the at least one intermediate compound and having a Formula VII and wherein A is a leaving group



62. The method of claim 61, wherein A is selected from the group of leaving groups consisting of chloride, nitrophenoxy, imidazolyl, N-hydroxysuccinimidyl, N-hydroxybenzotriazolyl, N-hydroxyazobenzotriazolyl, pentafluorophenoxy and N-hydroxysulfosuccinimidyl.

63. The method of claim 61 or 62, wherein the activating agent is selected from the group of activating agents consisting of 4-nitrophenyl chloroformate or disuccinyl carbonate.

64. The method of one of claims 62 or 64, wherein the amine-containing biologically active molecule is attached to the compound of Formula VII by displacement of the leaving group A.

65. A method for hydrolysing the molecule of any one of claims 1 to 28 comprising a step of placing the molecule in solution with a pH of approximately 7.4

66. The method of claim 65, wherein the solution is an extra-cellular fluid.

67. Method of administration of a biologically active compound to a living organism comprising:  
- a first step of attaching an amine-containing compound through an amino group to a carrier molecule by means of a linker;

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- a second step of administering the amine-containing compound and the carrier molecule to the living organism; and
- a third step of cleaving the amine-containing compound from the carrier molecule by means of a substantially non-enzymatic reaction.

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68. The method of claim 67, wherein the amine-containing compound is a biologically active molecule.

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69. The molecule of claim 68 wherein the biologically active molecule is selected from the group of biologically molecules consisting of small molecule biologically active agents or biopolymers.

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70. The molecule of claim 68, wherein the biopolymers are selected from the group of biopolymers consisting of proteins, polypeptides, oligonucleotides and peptide nucleic acids.

20

71. The molecule of claim 70, wherein the polypeptides are selected from the group of polypeptides consisting of ACTH, adenosine deaminase, agalsidase, albumin, alfa-1 antitrypsin (AAT), alfa-1 proteinase inhibitor (API), alteplase, anistreplase, anrod serine protease, antibodies (monoclonal or polyclonal, and fragments or fusions), antithrombin III, antitrypsina, aprotinin, asparaginases, biphalin, bone-morphogenic proteins, calcitonin (salmon), collagenase, DNase, endorphins, enfuvirtide, enkephalins, erythropoietins, factor VIIa, factor VIII, factor VIIIa, factor IX, fibrinolysin, fusion proteins, follicle-stimulating hormones, granulocyte colony stimulating factor (G-CSF), galactosidase, glucagon, glucocerebrosidase, granulocyte macrophage colony stimulating factor (GM-CSF), phospholipase-activating protein (PLAP), gonadotropin chorionic (hCG), hemoglobins, hepatitis B vaccines, hirudin, hyaluronidases, iduronidase, immune globulins, influenza vaccines, interleukins (1 alfa, 1 beta, 2, 3, 4, 6, 10, 11, 12), IL-1 receptor antagonist (rhIL-1ra), insulins, interferons (alfa 2a, alfa 2b, alfa 2c, beta 1a, beta 1b, gamma 1a, gamma 1b), keratinocyte growth factor (KGF), transforming growth factors, lactase, leuprolide, levothyroxine, luteinizing hormone, Lyme vaccine, natriuretic peptide, pancrelipase, papain, parathyroid hormone,

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5 PDGF, pepsin, platelet activating factor acetylhydrolase (PAF-AH), prolactin, protein C, octreotide, secretin, sermorelin, superoxide dismutase (SOD), somatropins (growth hormone), somatostatin, streptokinase, sucrase, tetanus toxin fragment, tilactase, thrombins, thymosin, thyroid stimulating hormone, thyrotropin, tumor necrosis factor (TNF), TNF receptor-IgG Fc, tissue plasminogen activator (tPA), TSH, urate oxidase, urokinase, vaccines, and plant protein such as lectin and ricin.

10 72. The molecule of claim 70, wherein the protein is a protein prepared by recombinant DNA technology.

15 73. The molecule of claim 70, wherein the protein is selected from the group of proteins consisting of antibody fragments, single chain binding proteins, catalytic antibodies and fusion proteins.

20 74. The molecule of claim 69, wherein the small molecule biologically active agents are selected from the group of agents consisting of central nervous system-active agents, anti-infective, anti-neoplastic, antibacterial, anti-fungal, analgesic, contraceptive, anti-inflammatory, steroidal, vasodilating, vasoconstricting, and cardiovascular agents with at least one primary or secondary amino group.

25 75. The molecule of claim 69, wherein the small molecule biologically active agents are selected from the group of compounds consisting of daunorubicin, doxorubicin, idarubicin, mitoxantron, aminoglutethimide, amantadine, diaphenylsulfon, ethambutol, sulfadiazin, sulfamerazin, sulfamethoxazol, sulfalen, ciprofloxacin, moxifloxacin, ciprofloxacin, enoxacin, norfloxacin, neomycin B, spectinomycin, kanamycin A, meropenem, dopamin, dobutamin, lisinopril, serotonin, acivicin, and carbutamid.

30 76. The method of one of claims 67 to 75, wherein the third step is carried out in an extra-cellular fluid.

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77. The method of one of claims 67 to 76, wherein the substantially non-enzymatic reaction comprises a step of hydrolysis.
78. The method of one of claims 67 to 77, wherein the substantially non-enzymatic reaction comprises a step of intramolecular cyclization.
79. The method of one of claims 67 to 78, further comprising a step of sterically protecting at least part of the linker by a sterically demanding carrier.
80. In an amine-containing compound attached to a carrier by a linker, a method of cleaving the amine-containing compound from the carrier by a substantially non-enzymatic reaction of the linker.
81. The method of claim 80, wherein the substantially non-enzymatic reaction is carried out at a pH of approximately 7.4.
82. The method of claim 80 or 81, wherein the amine-containing compound attached to the carrier is cleaved in an extra-cellular fluid.
83. The method of one of claims 80 to 82, wherein the substantially non-enzymatic reaction comprises a step of hydrolysis.
84. The method of one of claims 80 to 83, wherein the substantially non-enzymatic reaction comprises a step of intramolecular cyclization.
85. The method of one of claims 80 to 84, further comprising a step of sterically protecting at least part of the linker by a sterically demanding carrier.
86. The method of one of claims 80 to 85, wherein the amine-containing compound is a biologically active compound.

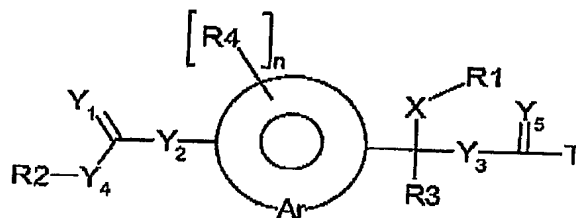
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### ABSTRACT

A molecule is disclosed having the following structure:

5



10 wherein

T is D or A

D being a residue of an amine containing biologically active molecule and  
A being a leaving group.



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Fig. 1

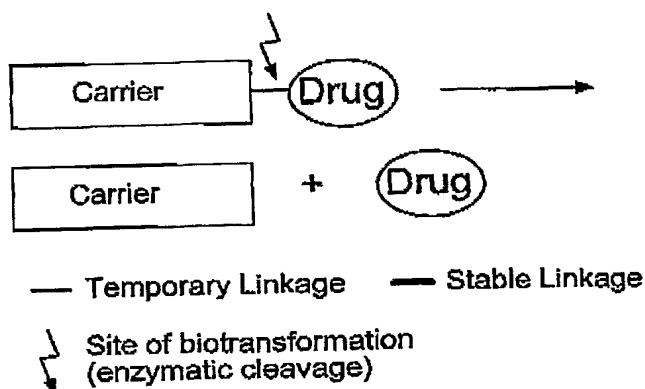


Fig. 2

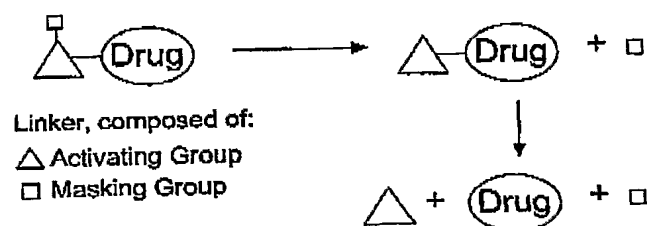
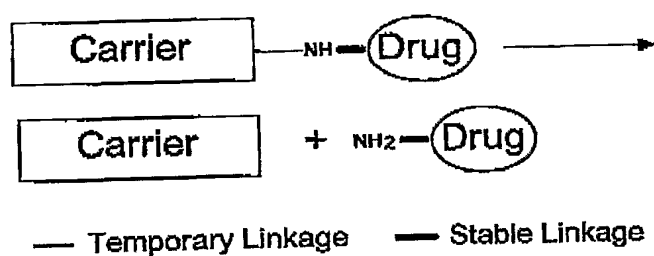


Fig. 3



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Fig. 4

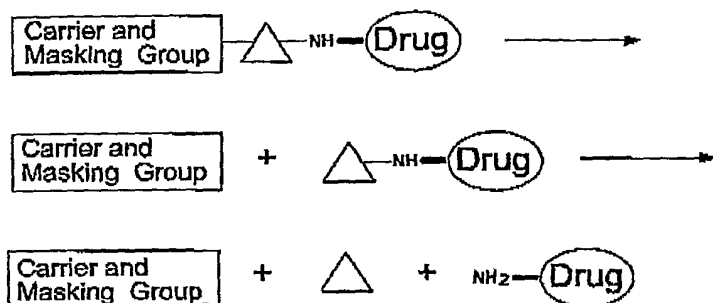
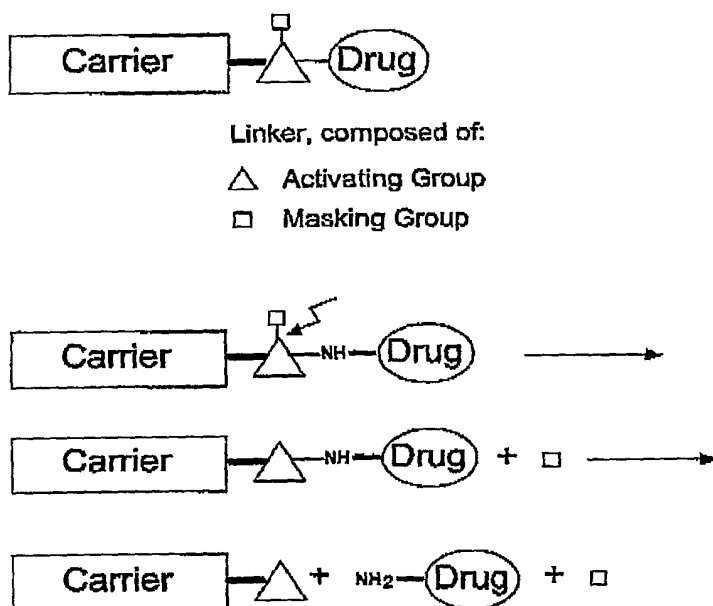


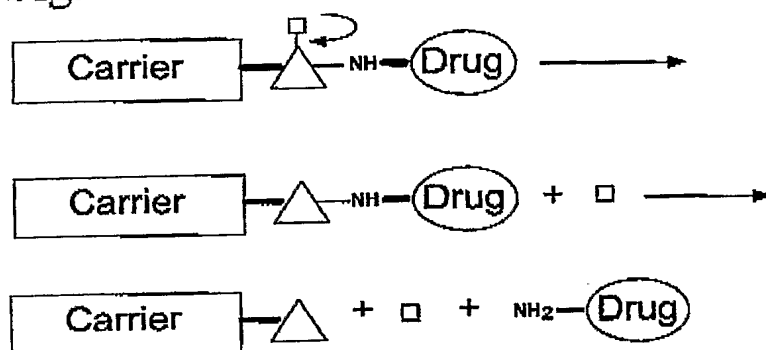
Fig. 5



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Fig. 6



↪ Site of Chemical Transformation  
(Non-Enzymatic Cleavage)

Fig. 7

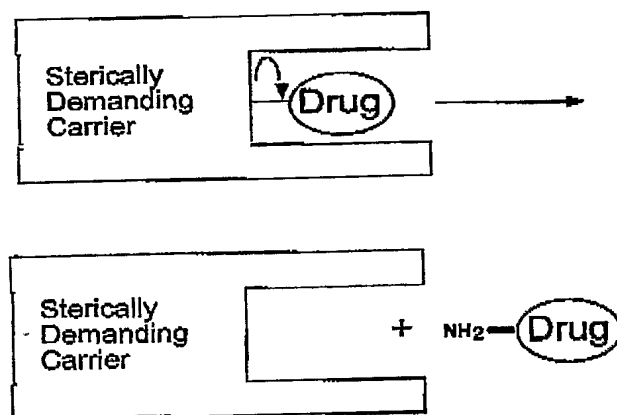
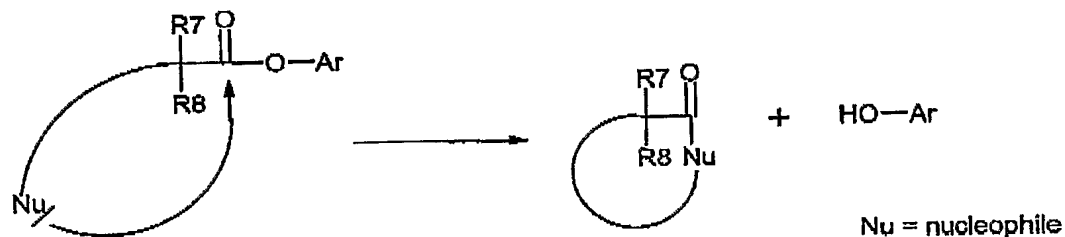


Fig. 8



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